A STUDY OF THE COLLAGEN AND ELASTIN CONTENT OF HUMAN LUNG PARENCHYMA IN RELATION TO AIRSPACE SIZE AND EMPHYSEMA

by

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Declaration of originality

I declare that, unless otherwise stated, this thesis represents my own work and was composed by me.

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ABSTRACT

A quantitative study of collagen and elastin content in human lung tissue has been made in relation to a morphometric measure of airspace size. The subjects included non-smokers, smokers with and without macroscopic emphysema and three subjects with alpha 1-protease inhibitor (α1-Pi) deficiency. In the selection of tissue samples, pleural and major bronchovascular structures were excluded, thereby limiting the study to changes in the acinar unit. Airspace size was determined morphometrically as Alveolar Wall surface area per Unit Volume of lung (AWUV). Total collagen was determined as hydroxyproline and elastin was determined as the specific cross-links desmosine and isodesmosine.

The results were as follows:

1. There were no significant differences in AWUV, collagen content or elastin content between the upper and lower lobes within a single lung from both a non-smoker and a smoker without macroscopic emphysema.

2. Analysis of 102 samples from 9 smokers' lungs with no signs of macroscopic emphysema showed significant negative correlations between AWUV and collagen content and between AWUV and elastin content such that as the surface area of alveolar wall per unit volume decreased there was an increase in both the collagen and elastin content of the remaining alveolar tissue. These results may reflect the heterogeneity in the collagen content, elastin content and airspace size of the various structures within the acinar unit, or, at least in the case of collagen, may reflect the presence of fibrotic tissue within the alveolar walls as a result of exposure to cigarette smoke.

3. In tissue samples from 14 non-smokers there was no significant correlation between age and collagen content or between AWUV and collagen content.

4. Samples taken from smokers' lungs where either macroscopic centriacinar emphysema or panacinar emphysema or a mixture of centriacinar and panacinar emphysema were present were found to have a significantly higher collagen content than samples from non-smokers. Samples from smokers' lungs without macroscopic emphysema also had a higher collagen content than samples from non-smokers though this was not statistically significant. This increased collagen may represent fibrotic tissue in thickened alveolar walls as a result of inflammatory injury caused by cigarette smoke followed by fibrotic healing.

5. Tissue samples from the lungs of three α1-Pi deficient subjects had a significantly higher collagen content than samples from a non-smoker, though there were no significant differences in elastin content. The increase in collagen content is consistent with the presence of fibrotic lung tissue.

In view of these findings the definition of emphysema, which states that no obvious fibrosis is present, may have to be revised. The protease-antiprotease hypothesis of emphysema, which attributes the loss of alveolar wall in emphysema to a loss of elastin, may also have to be reconsidered.

LIST of CONTENTS

ABBREVIATIONS

The following is a list of abbreviations which are used in the main text of the thesis.

AMINO ACIDS

Single letter code	Three letter code	Amino acid
A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
P	Pro	proline
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
Y	Tyr	tyrosine
	Asn	asparagine
	Hyp	4-hydroxyproline
	3,4-DHP	3,4 dehydroproline
	Nor	nor-leucine
	DES	desmosine
	ISO	isodesmosine

OTHER ABBREVIATIONS

α1-Pi	alpha 1 - protease inhibitor
ANOVA	analysis of variance
AWUV	alveolar wall surface area per unit volume of inflated lung
B/B0%	amount of HRP-coupled second antibody which is bound, expressed as a percentage of that bound in the absence of soluble antigen
COL	collagenous
COPD	chronic obstructive pulmonary disease
DIEA	diisopropylethylamine
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
FACIT	fibril-associated collagens with interrupted triple helices
FIP	Fast Interval Processor
FMOC-Cl	9-fluorenylmethylchloroformate
H & E	haematoxylin and eosin
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
hr	hour (s)
ID	internal diameter
Lm	mean linear intercept
mA	milliamp
min	minute (s)
MPO	myeloperoxidase
NC	non-collagenous
NMR	nuclear magnetic resonance
OPA	orthophthaldialdehyde
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PITC	phenylisothiocyanate
PTC-AA	phenylthiocarbamyl-amino acid
RNA	ribonucleic acid
s	second (s)
SD	standard deviation
SDS	sodium dodecyl sulphate
Suc NCl	N-chlorosuccinimide

TEMED	tetramethylethylenediamine
v / v	volume per volume
w / v	weight per volume
yr	year (s)

CHAPTER 1. INTRODUCTION

1.1 The Structure and Function of the Respiratory Tract.

The main function of the respiratory tract is to bring atmospheric oxygen into close association with venous blood. The oxygen then diffuses into the blood and is carried on haemoglobin to all the tissues of the body. Similarly waste carbon dioxide is removed from the body when it diffuses from the blood into the respiratory tract. Other functions include elimination of inhaled particles and organisms, filtration of particulate matter from the circulation and metabolism of certain drugs and enzymes. The lungs also serve as a route of administration of anaesthetic and other drugs and have a role in water balance and the maintenance of blood pH (Seaton *et al.*, 1989).

Efficient gas exchange is achieved by virtue of the large surface area of the internal epithelium of the lungs. However, this also results in increased opportunities for damage to the lungs by organisms, particles and gases and for absorption into the body of harmful substances.

The upper respiratory tract, which includes the nose, the pharynx, the paranasal sinuses, the eustachian tube and the larynx, has several functions besides air conduction, including swallowing, air conditioning, smell and speech.

The lower respiratory tract begins where the trachea extends from the larynx. The trachea runs down to the level of the fifth thoracic vertebra and averages about 10-12 cm in the adult human. At its lower end the trachea divides into the right and left main bronchi. The right main bronchus divides into the right upper lobe bronchus, which in turn divides into anterior, apical and posterior segmental bronchi, and the bronchus intermedius. This latter gives off middle lobe and apical lower lobe bronchi, then divides into the four basal segmental bronchi, anterior, medial (cardiac), posterior and lateral. The middle lobe bronchus divides into medial and lateral segmental bronchi. The left main bronchus gives off upper and lower lobe bronchi; the upper lobe bronchus divides into apico-posterior and anterior segmental bronchi and a lingular bronchus that in turn divides into superior and inferior bronchi. The left lower lobe bronchus gives off an apical bronchus and then divides into anterior, lateral and posterior segmental bronchi. There is no left medial basal segmental bronchus. Anomalous bronchi are, however, relatively frequent (Seaton *et al.*, 1989; Flenley, 1990).

The trachea, main bronchi and lower lobe bronchi are outside the lung. All other bronchi are situated within the lung.

The lower airways are known as bronchi down to the smallest divisions containing cartilage, however sparse. They then become bronchioles, the final branch of this type being the terminal bronchiole. Subsequent divisions contain increasing numbers of alveoli in their walls and are called respiratory bronchioles; these give off the alveolar ducts which then give off the alveolar sacs. The lung unit from each terminal bronchiole is called the acinus (Figure 1.1).

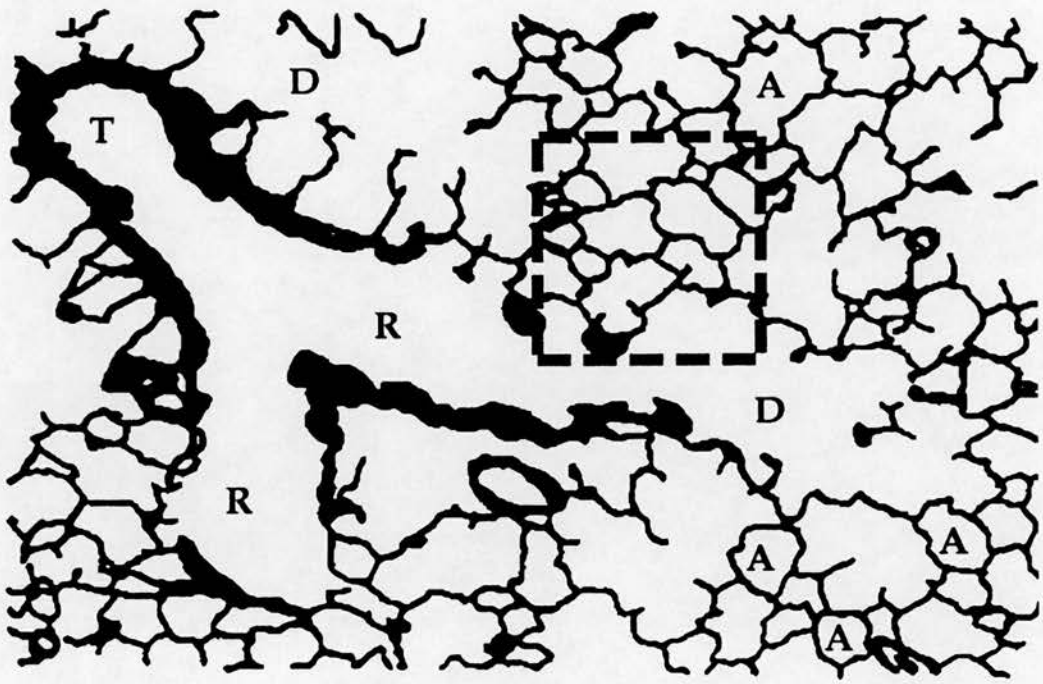


Figure 1.1 Digital binary image from a histological section of human lung parenchyma showing the successive change in structure from proximal to distal end of the acinar unit. The dashed box represents $1 mm^2$ of tissue. Magnification x 40. Kindly supplied by Dr. M.R. Lang, Department of Pathology, University of Edinburgh. T = terminal bronchiole; R = respiratory bronchiole; D = alveolar duct; A = alveolar sac.

From the smallest bronchi there are about 3-4 further sub-divisions of bronchioles before the terminal one is reached. There are about 25,000 terminal bronchi, each of which divides into two respiratory bronchioles (Horsfield, 1981). There are usually two subsequent divisions of respiratory bronchioles, the more peripheral branches containing more alveoli, and the final division is into alveolar ducts which are completely surrounded by alveoli. Up to nine generations of alveolar ducts occur before the alveolar sacs arise as the terminal unit of the airways. There are about 28 orders of division of the tracheobronchial tree. The total number of alveoli has been estimated to be between 2 and 6 x 10^8 (Angus and Thurlbeck, 1972; Hansen and Ampaya, 1975).

One side of each respiratory bronchiole is devoid of alveoli and in their place runs a pulmonary artery. These vessels give off arteriolar branches which in turn supply the capillaries to alveoli. Each alveolus is surrounded by a network of capillaries with very thin walls composed of endothelial cells. The alveolar wall thus consists of a layer of alveolar epithelial cells (Type I and Type II pneumocytes), their basement membrane, a thin interstitial space which contains collagen and elastin, unmyelinated nerves and occasional macrophages, the capillary basement membrane and capillary endothelial cells (Corrin, 1981). In many places the basement membranes fuse, minimising the distance for gas diffusion from alveolar space to capillary to about 0.2 μm. The capillaries then join to form venules which in turn become pulmonary veins.

The pleura, which covers the lung, consists of a single layer of mesothelial cells, without basement membrane. A layer of connective tissue separates it from the adipose tissue of the chest wall and from alveoli.

1.2 Emphysema.

The National Heart, Lung and Blood Institute, at their workshop on the definition of emphysema (Snider *et al.*, 1985), reaffirmed the 1962 American Thoracic Society definition of emphysema, but with the qualification that obvious fibrosis should be absent. Emphysema was defined as "a condition of the lung characterised by abnormal permanent enlargement of air spaces distal to the terminal bronchioles accompanied by destruction of their walls and without obvious fibrosis". Destruction was defined as "nonuniformity in the pattern of respiratory airspace enlargement so that the orderly appearance of the acinus and its components is disturbed and may be lost". An acinus is defined as "the respiratory airspaces arising from a single terminal bronchiole". Destruction may be recognised by the naked eye, by subgross examination (low power magnification) of an inflation-fixed lung slice,

or by light microscopic evaluation of thick (200 - 400 μm) or thin (4 - 6 μm) stained and mounted sections.

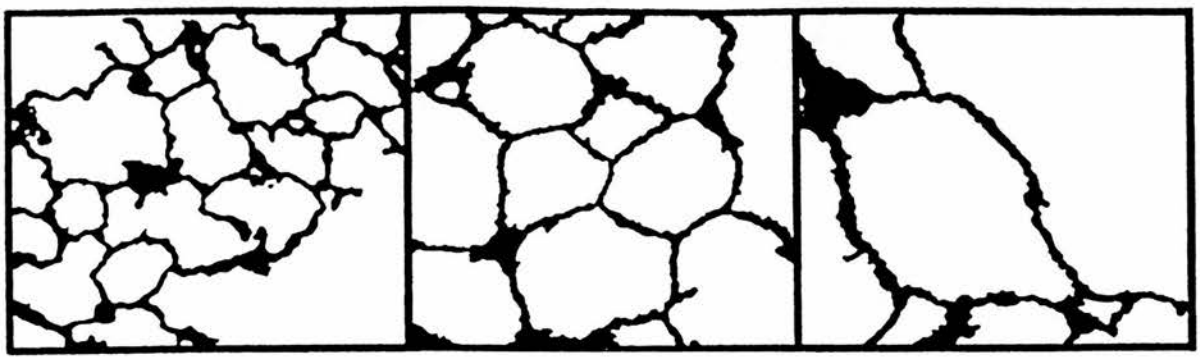
Three major anatomic subtypes of emphysema have been described based on the portions of the acinus primarily involved, as observed in mild disease. As emphysema becomes more severe, classification into the anatomic subtypes becomes difficult. The three anatomic subtypes are:

a. Centriacinar emphysema (Leopold and Gough, 1957; Kuhn and Askin, 1985). Centriacinar emphysema is characterised by selective and dominant involvement of the respiratory bronchioles with subsequent extension of the process to the alveoli. Two different forms are known. The first of these is classically associated with cigarette smoking and air-flow obstruction and is known as centrilobular emphysema. The lesion usually begins and is most severe in the upper lung. The second form is associated with exposure to coal and other mineral dust and also results in dilation of respiratory bronchioles with intense accumulations at those sites of dust-laden macrophages. The lesion is relatively uniformly distributed in the lungs, and has been termed focal emphysema. In those exposed to coal dust, the term coal pneumoconiosis is used.

b. Panacinar emphysema (Wyatt *et al.*,1962; Eriksson, 1965; Thurlbeck, 1976; Kuhn and Askin, 1985). Panacinar emphysema, also known as panlobular emphysema, tends to involve all of the acinus uniformly. The process tends to be worse at the base of the lung and to accompany the centrilobular emphysema of cigarette smokers. As emphysema becomes severe, it is increasingly difficult to distinguish between centrilobular and panacinar emphysema. Some pathologists feel that centrilobular emphysema progresses into panacinar emphysema. Panacinar emphysema is commonly observed with homozygous alpha 1-antitrypsin deficiency.

c. Distal acinar emphysema. In distal acinar emphysema, the alveolar ducts and sacs are predominantly involved.

It has been shown that there is an age related increase in airspace size in non-smokers, such that between one-third (Thurlbeck, 1967b) and one-quarter (Gillooly and Lamb, 1993a) of the total alveolar walls are lost between the ages of 20 - 80 years (Figure 1.2). This increase in airspace size is not regarded as emphysema but rather as a normal increase associated with advancing age in adult lungs.



22 year old non-smoker | 82 year old non-smoker | 70 year old smoker with panacinar emphysema

Figure 1.2 Digital binary images from histological sections of human lung parenchyma demonstrating the enlargement of airspace size with age and as a result of panacinar emphysema. Magnification x 40. Kindly supplied by Dr. M.R. Lang, Department of Pathology, University of Edinburgh.

The association between homozygous alpha 1-antitrypsin deficiency and pulmonary emphysema (Laurell and Eriksson, 1963) was dicovered at about the same time as the work of Gross *et al.* (1964) on the experimental model of papain-induced emphysema. The logical connection between these two independent studies was the theory that emphysema might be due to an imbalance between naturally occurring proteases and antiproteases. Much of the rationale for the experimental work done since then arose from this concept.

Gross *et al.* (1964) were the first to produce an experimental model of emphysema which bore any resemblance to human centrilobular emphysema. They did this in rats by the intratracheal injection of the proteolytic enzyme papain. They were attempting to ameliorate the fibrotic response of rat lungs to quartz dust exposure, but instead noted the development of a patchy air-space lesion that developed within hours after instillation of papain. Destruction of respiratory bronchioles and alveolar ducts was believed to have occurred before alveolar wall disruption.

Johanson *et al.* (1973) exposed rats to an aerosol of 10% papain for four hours. They found that papain selectively attacked the amorphous component of elastic fibres, leaving the micro-fibrils intact. Collagen was not altered histologically. Connective tissues appeared normal in animals examined later than four weeks after exposure. Taken together with the findings of Pierce *et al.* (1961), that the lung content of collagen and elastin in human emphysema was not significantly different from normal, the observations of Johanson *et al.* (1973) led these authors to propose a pathogenic mechanism for the development of emphysema that involved acute dissolution of elastin by proteolytic enzymes, followed by structural remodelling of the lung with regeneration on the persisting microfibrillar skeleton. Following on from this, Kaplan *et al.* (1973) produced emphysema in Syrian hamsters by an intratracheal injection of porcine pancreatic elastase. The induction of emphysema was inhibited by mixing the elastase with normal serum but not by mixing with serum deficient in alpha 1-antitrypsin. The fact that another elastase occurs in granules of both neutrophil polymorphonuclear leucocytes (neutrophils) and macrophages means that it is available to act within the lung. Marco *et al.* (1971) demonstrated that aerosolised homogenates of neutrophils and macrophages produced emphysema in dogs. Janoff *et al.* (1977) showed, by an immunoperoxidase technique, the direct attachment of human neutrophil elastase to elastic fibres within alveolar walls. In enzyme treated tissue, some alveolar walls showed severe depletion of intercellular structures with the exception of collagen, which was generally preserved.

Habitual cigarette smoking is probably the single most important aetiologic co-factor in the pathogenesis of human emphysema. As early as 1964, Anderson *et al.* showed a strong relationship between centrilobular emphysema and smoking in 71 autopsies. No significant relationship was found between smoking and panacinar emphysema. The importance of smoking and age in the development of centrilobular emphysema has been confirmed by Thurlbeck *et al.* (1974), who looked at random necropsies from three populations in Canada, Wales and Sweden and found that the severity of emphysema was related to cigarette smoking. Age and sex also played a role, with women having less emphysema than men when normalised for age and smoking history.

Janoff and Carp (1977), in their search for a mechanism by which cigarette smoking might favour excess of free proteases in the lung, showed that whole, unfractionated cigarette smoke suppressed the antiprotease activity of normal human serum, pure human alpha 1-antitrypsin and human bronchopulmonary lavage fluid. Kilburn and McKenzie (1975) showed that cigarette smoke recruited neutrophils from the trachea to the terminal bronchioles of hamster lungs. Neutrophil and macrophage numbers are also increased in the lungs of cigarette smokers compared to non-smokers (Pratt *et al.*, 1969; Niewoehner *et al.*, 1974; Hunninghake and Crystal, 1983). It has also been shown that cultured alveolar macrophages release factors that are chemotactic for neutrophils (Hunninghake *et al.*, 1980; Merrill *et al.*, 1980; Cohen *et al.*, 1982). Hunninghake and Crystal (1983) demonstrated that human alveolar macrophages secrete neutrophil chemotactic factors when exposed to cigarette smoke *in vitro,* and that unstimulated macrophages from smokers release more of these factors than do cells from non-smokers. Nicotine, at high concentrations, has also been shown to be a chemoattractant for neutrophils, whereas at more physiological concentrations it enhances the chemotactic responsiveness of neutrophils to C5a, a chemotactically active peptide generated during activation of the complement cascade (Totti *et al.*, 1984). Once inflammation has been initiated by cigarette smoke, products of proteolysis might serve to maintain the inflammatory reaction. Proteolytic digestion products of both elastin and collagen have been shown to have chemotactic activity for neutrophils *in vitro* (Chang and Houck, 1970; Senior *et al.*, 1980).

By the late 1970s and early 1980s, therefore, data had accumulated suggesting an inflammatory process, resulting from cigarette smoke, in the pathogenesis of emphysema. Cigarette smoke had been shown to attract neutrophils and alveolar macrophages into the lung and thereby increasing the protease burden in the lung, in particular elastase. Cigarette smoke had also been shown to reduce the

anti-protease activity of alpha 1-antitrypsin, the natural defense against elastase. The question, however, remained as to the mechanism by which cigarette smoke inactivates alpha 1-antitrypsin. Alpha 1-antitrypsin inactivates serine proteinases of vastly different specificities by forming 1 : 1 molar complexes which are stable even in the presence of sodium dodecyl sulphate (Pannell *et al.,* 1974). Johnson and Travis (1978) showed that the reactive centre of human alpha 1-antitrypsin contains a methionine residue. They later tested the importance of this residue by oxidising the inhibitor with increasing concentrations of N-chlorosuccinimide (SucNCl; Johnson and Travis, 1979). A maximum of two of the eight methionine residues in the alpha 1-antitrypsin molecule were modified, even at very high levels of oxidising agent (40 mol of SucNCl/mol of inhibitor). Over 95% inhibitory activity towards porcine trypsin, porcine pancreatic elastase, and human neutrophil elastase was lost when the two methionine residues were oxidised. However, if the oxidised inhibitor was incubated with the three proteinases for extended periods of time much of the inhibitory activity towards porcine trypsin and human neutrophil elastase was regained but there was no inhibition of porcine elastase. N-terminal sequence analysis of a peptide fragment obtained from a papain digest of oxidised alpha 1-antitrypsin and purified by gel filtration and shown to contain the reactive site (Johnson and Travis, 1978) confirmed that the reactive centre methionine had been oxidised. Gel electrophoresis revealed that trypsin and neutrophil elastase complexes with oxidised alpha 1-antitrypsin were unstable after treatment with SDS. Johnson and Travis (1979) concluded that it was quite likely that cigarette smoke contains, or is capable of forming, oxidising agents which inactivate alpha 1-antitrypsin through conversion of the reactive centre methionine to the sulphoxide or sulphone form, thereby reducing the proteinase inhibitor activity that is normally present in the lung. A second source of oxidants may be due to accidental biological oxidation. Myeloperoxidase (MPO), an enzyme present in large amounts in human neutrophils acts together with hydrogen peroxide (H_2O_2) and a halide to generate potent oxidants capable of reacting with a variety of cellular and humoral targets (Clark, 1983). Although MPO serves its primary role as a microbicidal agent within phagocytic vacuoles, it is released, along with H_2O_2, into extracellular fluid during phagocytosis. Phagocytic cells, including neutrophils, are present in increased numbers in the lungs of cigarette smokers. Several workers have now shown that the elastase-inhibitory capacity of alpha 1-antitrypsin is lost when exposed either to a cell-free MPO - H_2O_2 - halide system or to human neutrophils, both of which result in a loss of the ability of the inhibitor to bind and therefore inactivate human neutrophil elastase (Carp and Janoff, 1980; Clark *et al.,* 1981; Matheson *et al.,* 1981; Zaslow *et al.,* 1983).

The neutrophil is now thought to be the most likely source of elastase in the pathogenesis of human emphysema. Alveolar macrophages are thought to be a source of neutrophil chemotactic factor and a possible endogenous source of elastase (White *et al.*, 1977; Campbell *et al.*, 1979; Banda *et al.*, 1980; McGowan *et al.*, 1983). In summary the protease-antiprotease hypothesis of the pathogenesis of human emphysema in cigarette smokers is as follows: cigarette smoke attracts alveolar macrophages to cluster around respiratory bronchioles (Niewoehner *et al.*, 1974) with a release of neutrophil chemotactic factor, and possibly elastase. Neutrophils are recruited to the area from the blood and release elastase. Alpha 1-antitrypsin (also known as alpha 1-protease inhibitor, α1 - Pi), the natural defense against proteases, is rendered inactive by oxidation from oxidants present in cigarette smoke or from the myeloperoxidase system of neutrophils or indeed both. Elastase is then left, unimpaired, to breakdown the elastin fibre network ultimately leading to a permanent loss of alveolar architecture.

1.3 Collagen.

1.3.1 Introduction.

The collagens are a family of proteins integral to the structure and function of the extracellular matrix (Table 1.1). Some collagens represent the most abundant proteins in a variety of vertebrate tissues, including lung. At least fourteen different types have been discovered forming a wide range of structures in a range of tissue types (for reviews see van der Rest and Garrone, 1991; Hulmes, 1992). Collagens of various types are the major group of proteins in the lung and are present in all the major structures, including airways, blood vessels, parenchyma and the basement membranes of epithelial and endothelial cells.

Common to all collagens is the presence of one or several domains that have a characteristic triple helical conformation. The amino acid sequences of the triple helical domains are characterised by a repeating triplet amino acid sequence, X-Y-Gly, where about 30% of the X and Y positions are occupied by proline and hydroxyproline residues, respectively.

The collagen triple helix is composed of three polypeptide chains (α chains) where each chain is itself a left-handed helix (Ramachandran and Kartha, 1954; 1955). Each chain assembles with two other chains in a right-handed super-helix in which every glycine residue is buried along the axis of the helix (Traub and Piez, 1971; Piez and Miller, 1974). Since glycine is the only amino acid with no side chain, there is an absolute requirement that it occupies every third position. Any other amino acid

Table 1.1 Vertebrate collagens.

Class	Type	α chains	Most common molecular form	Tissue distribution
Fibrillar	I	α1(I),α2(I)	$[\alpha1(I)]_2\ \alpha2(I)$	Most connective tissues eg. bone, tendon, skin, lung, cornea, sclera, vascular system.
	II	α1(II)	$[\alpha1(II)]_3$	Cartilage, vitreous humour, embryonic cornea.
	III	α1(III)	$[\alpha1(III)]_3$	Extensible connective tissue eg. skin, lung, vascular system.
	V	α1(V), α2(V), α3(V)	$[\alpha1(V)]_2$ α2(V)	Tissues containing collagen I.
	XI	α1(XI), α2(XI), α3(XI)	α1(XI) α2(XI) α3(XI)	Tissues containing collagen II.
Network forming	IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV)	$[\alpha1(IV)]_2\ \alpha2(IV)$	Basement membranes.
Fibril associated	IX	α1(IX), α2(IX), α3 (IX)	α1(IX) α2(IX) α3(IX)	Tissues containing collagen II.
	XII	α1(XII)	$[\alpha1(XII)]_3$	Associated with collagen I in tendon and ligament.
	XIV	α1(XIV)	$[\alpha1(XIV)]_3$	Skin and tendon.
Short chain collagens	VIII	α1(VIII), α2(VIII)	?	Descemet's membrane.
	X	α1(X)	$[\alpha1(X)]_3$	Hypertrophic zone of cartilage.
Filamentous	VI	α1(VI), α2(VI), α3(VI)	α1(VI) α2(VI) α3 (VI) α3(VI)	Most connective tissues.
Long Chain	VII	α1(VII)	$[\alpha1(VII)]_3$	Associated with the basement membrane of certain epithelial cell layers eg. at the dermal-epidermal junction in skin.
Collagen XIII	XIII	α1(XIII)	$[\alpha1(XIII)]_3$?	Skin and intestine

Collagen molecules are composed of three α chains. Different chains within a single molecule are designated using Arabic numerals, while different collagen types are designated using Roman numerals.

would perturb the triple helical conformation (Ramachandran and Reddi, 1976). Amino acids in the X and Y positions have their side chains directed outward where they can participate in lateral interactions, particularly with other triple helices.

The collagen types have been grouped according to their appearance in the electron microscope. Collagens I, II III, V and XI appear as striated fibrils and are therefore referred to as the fibrillar collagens. The banding pattern is a result of the aligment of staggered 300 nm collagen molecules with gaps and overlaps (Hodge and Petruska, 1963). Fibrils are formed as a result of lateral interaction between homologous regions within the triple helical domains (Hulmes *et al.*, 1973). The molecules are staggered by approximately 67 nm (the D-stagger). Fibrils of more than one collagen type have been shown to occur *in vivo* (Henkel and Glanville, 1982; Keene *et al.*, 1987; Birk *et al.*, 1988; Mendler *et al.*, 1989).

1.3.2 Biosynthesis of Fibril-Forming Collagens.

The fibrillar collagens are first synthesised as precursor prepro α chains containing N-terminal hydrophobic pre-peptide (or signal) sequences. The pre-peptide probably binds to the membrane of the rough endoplasmic reticulum (E.R.) and leads to the translocation of the prepro α chains into the cisternae of the rough E.R. (Blobel *et al.*, 1979; Davis and Tai, 1980). The signal sequence is then removed during or shortly after translocation. In the rough E.R. cisternae the pro α chains undergo extensive processing by post-translational modifications. Certain proline and lysine residues are hydroxylated by prolyl 4-hydroxylase, to a lesser extent by prolyl 3-hydroxylase, and by lysyl hydroxylase, respectively (Kivirikko and Myllyla, 1985; Kivirikko *et al.*, 1989, 1990). The presence of hydroxyproline residues provides critical stability to the triple helix of the collagen molecule which would otherwise be unstable at body temperature (Prockop *et al.*, 1979). Most of the hydroxylation occurs while the nascent polypeptide chains are growing on the ribosomes. Hydroxylation continues, however, after release of complete polypeptide chains from the ribosomes into the cisternae of the rough E.R., until triple helix formation of the pro α chains prevents any further hydroxylation. Prolyl 4-hydroxylase requires an X-Pro-Gly sequence and lysyl hydroxylase an X-Lys-Gly sequence (Kivirikko and Myllyla, 1985; Kivirikko *et al.*, 1989, 1990). As a result 4-hydroxyproline and hydroxylysine occupy almost exclusively the Y position of the X-Y-Gly sequence. Prolyl 3-hydroxylase requires a Pro- 4-hydroxyproline -Gly sequence (Risteli *et al.*, 1977). The exception to this rule occurs in the non-triple-helical extensions at both the amino (N) and carboxy (C) terminals. In both these regions, a single lysine residue, not followed by a glycine, is frequently

hydroxylated. Certain hydroxylysine residues are further modified by glycosylation, both co-translationally and post-translationally (Kivirikko and Myllyla, 1979) O-Glycosylation of hydroxlysine is catalysed by two specific enzymes: **(1)** Galactosylhydroxylysyltransferase, which transfers galactose from UDP-galactose to peptidyl hydroxylysine, forming O-α-D-galactosylhydroxylysine, and **(2)** glucosylgalactosylhydroxylysyltransferase, which transfers glucose from UDP-glucose to galactosylhydroxylysine, forming O-β-D-(2-α-D-glucosyl)-galactosylhydroxylysine. The extent of glycosylation is variable for the different genetic types of collagen and in the same collagen type from different tissues in the same organism.

The C-propeptides of procollagen contain asparagine-linked carbohydrate units that are not present in the collagen domain of the molecule (Olsen *et al.*, 1977; Clark, 1979; Torre-Blanco *et al.*, 1992). A mannose-rich oligosaccharide side chain is transferred to an asparagine residue in the -Asn-X-Ser/Thr-sequence (Olsen *et al.*, 1977; Clark, 1979; Torre-Blanco *et al.*, 1992).

An important step prior to triple helix formation is association and registration of the three chains through the globular C-terminal domains. Accompanying these events is the formation of intra- and interchain disulphide bonds, catalysed by the enzyme protein disulphide isomerase. Triple helix formation then proceeds, in a zipper-like fashion, from the C-terminus to the N-terminal domain (Engel and Prockop, 1991). It is thought that triple helix formation occurs within the cisternae of the rough E.R. However, the possibility exists that the triple helix may be formed only as the pro α chains reach the Golgi apparatus (Prockop *et al.*, 1979).

All the fibrillar procollagens have the same overall structure (Mayne and Burgeson, 1987). In addition to the main triple helical domain of about 337 Gly-X-Y repeats, propeptides are present at both ends of the molecule. At the carboxyl end, the disulphide-bonded globular portion (the C-propeptide) is separated from the triple helix by a short linear domain which contains the C-proteinase cleavage site. At the amino end, the N-propeptide is usually composed of three structural regions: a highly variable globular region, a central short triple helical domain and a short linear domain that connects the peptide to the main triple helix and which contains the N-proteinase cleavage site. The globular region is absent in the pro α 2 (I) chain and is up to 383 residues in the pro α 1 (XI) chain (Yoshioka and Ramirez, 1990).

A number of possible functions have been suggested for the propeptides (Prockop *et al.*, 1979). As already discussed, the C-propeptides probably direct chain association. Both propeptides may also prevent premature fibril formation,

control fibrillogenesis, increase the rate and efficiency of folding of the pro α chains into a triple helix and, after they have been cleaved, provide a negative feedback mechanism to inhibit procollagen synthesis. Evidence suggests that both the C-propeptide and the N-propeptide may function as a negative feedback inhibitor of collagen biosynthesis (Wu *et al.*, 1986, 1991; Bornstein and Sage, 1989). They may also facilitate intracellular transport and packaging of procollagen into secretory granules.

The procollagen molecules are secreted into the extracellular space from secretory vesicles following packaging in the Golgi apparatus (Olsen, 1991). During secretion, or immediately following it, the N- and C-propeptides are enzymically removed by the procollagen N- and C-proteinases (Hojima *et al.*, 1989). The removal of the C-propeptide is necessary for fibril formation, whereas molecules with intact N-propeptides can still form fibrils, but they are smaller in diameter than the fibrils formed from fully processed collagen molecules (Fleischmajer *et al.*, 1983; Hulmes, 1983; Chapman, 1989). The enzymes are neutral proteinases and require calcium for maximal activity. The N-propeptides of both type I and type II procollagen are cleaved by the same enzyme (type I N-proteinase; Hojima *et al.*, 1989). The enzyme requires the triple helical conformation for maximal activity. Synthetic peptides with identical sequences to the cleavage site in pro α 1 (I) chains are not cleaved by the enzyme (Morikawa *et al.*, 1980). A different calcium dependent N-proteinase cleaves the N-propeptide of type III procollagen (Nusgens *et al.*, 1980; Halila and Peltonin, 1984, 1986). A C-proteinase, which cleaves the C-propeptide of types I, II and III procollagens, has been purified from several tissue sources (Hojima *et al.*, 1985; Kessler *et al.*, 1986). It also has a neutral pH optimum and requires calcium for maximal activity. A glycoprotein, which has been purified from mouse fibroblast culture medium and which is devoid of procollagen processing activity itself, has been shown to enhance the activity of C-proteinase (Kessler and Adar, 1989; Kessler *et al.*, 1990).

The collagen molecules produced by cleavage of procollagen spontaneously assemble into fibrils that are microscopically indistinguishable from mature fibrils found in tissues. The immature fibrils, however, lack tensile strength until they are cross-linked by a series of covalent bonds (Prockop *et al.*, 1979). Cross-linking of newly synthesised collagen fibrils is catalysed by the copper dependent enzyme lysyl oxidase (Kagan and Trackman, 1991). Lysyl oxidase catalyses the oxidative deamination of the ε amino groups of certain lysine and hydroxylysine residues to the corresponding aldehydes allysine and hydroxyallysine, respectively. These aldehydes spontaneously condense with non-oxidised or oxidised lysines (or hydroxylysines)

to form various Schiff base and aldol condensation cross-links, respectively. Collagens I, II and III contain four sites involved in cross-linking. Two sites are at the end of the molecule in the non-triple helical regions (telopeptides) and two are towards each end of the triple helix. An allysine or hydroxyallysine in the telopeptide of one collagen molecule reacts spontaneously with a lysine or hydroxylysine in the triple helix of an adjacent molecule in a head to tail configuration, thus stabilising the D stagger. Initially, a number of bi-functional cross-links are formed. As collagen matures a variety of tri-functional cross-links are formed (Eyre *et al.*, 1984; Last *et al.*, 1990).

1.3.3 Non-fibril Forming Collagens.

1.3.3.1 Collagen IV.

Collagen IV, one of a number of non-fibrillar collagens, is a major component of basement membranes. Basement membranes are sheet-like structures composed of laminin, heparan sulphate proteoglycans, nidogen (entactin) and BM-40 (osteonectin, SPARC) entrapped in a network of collagen IV (Timpl, 1989). They are produced by epithelial, endothelial, and many mesenchymal cells and provide a number of functions including support for cells and cell layers, selective molecular sieves such as the glomerulus of the kidney, and between tissue compartments. They also act to impede inflammatory and tumour cells and are involved in cell attachment, growth and differentiation (Yurchenco and Schittny, 1990).

The collagen IV molecule which has been characterised extensively is a heterotrimer consisting of two α 1 (IV) chains and one α 2 (IV) chain. Evidence exists for the presence of three additional chains (α 3 (IV), α 4 (IV) and α 5 (IV)), although it is not known whether they can form heterotrimers with either the α 1 (IV) or α2 (IV) chains (Saus *et al.*, 1988; Hostikka *et al.*, 1990). Their molecular assemblies are also not known. The collagen IV molecule measures approximately 400 nm in length and possesses a globular, non-collagenous (NC), domain at its C-terminus (NC1) and a short 30 nm triple helical segment at the N-terminus (the 7S domain) which has a role in oligomer formation. Unlike the fibrillar collagens, the molecule is not proteolytically modified before self-assembly. The main triple-helix also differs from the fibrillar collagens in that it is interrupted by short non-triple helical sequences. In human collagen IV there are 21 interruptions in the α 1 (IV) and 23 in the α 2 (IV) chain and these match each other in location in most cases to give a total of 25, fairly evenly distributed, interruptions along the helix (Timpl, 1989). Electron microscopy has shown that these interruptions impart increased flexibility to the molecule (Hofmann *et al.*, 1984). A characteristic feature

of the triple-helical domain is the presence of seven or eight Cys residues, mainly located in the non-helical sequences and in the 7S domain, which are involved in intra- and inter-molecular cross-links (Timpl, 1989). The α 1 (IV) and α 2 (IV) segments of the globular NC1 domain each have six Cys residues. Each set of Cys residues forms three disulphide bonds to give two symmetrical sub-domains within the NC1 segment (Siebold *et al.*, 1988).

Collagen IV self-assembles into a stable three-dimensional network using three types of interaction. Dimers can be generated by interactions between the NC1 domains while tetramers are formed by the lateral association of 7S segments which are aligned alternatively in parallel and antiparallel fashion. The dimers are stabilised through disulphide exchange between corresponding Cys residues of two monomeric domains (Siebold *et al.*, 1988). The tetramers are stabilised through disulphide bonds and non-reducible lysine and/or hydroxylysine derived cross-links. A further function of the NC1 domain may include the selection and alignment of newly synthesised α1 (IV) and α2 (IV) chains, since evidence suggests that folding of the triple helix starts from its C-terminus (Dölz *et al.*, 1988). The third type of interaction occurs through the lateral association of two dimers (Yurchenco and Schittny, 1990), to form an irregular polygonal network.

1.3.3.2 FACIT Collagens.

Collagens IX, XII and XIV constitute a group of fibril-associated collagens with interrupted triple helices (FACITS, Gordon and Olsen, 1990). They do not form fibrils themselves, but are specifically associated with certain fibrillar collagens. The best studied of this group is collagen IX which is associated with collagen II (Mayne and Burgeson, 1987). Collagen IX is a heterotrimer composed of three distinct chains, α 1 (IX), α 2 (IX) and α 3 (IX). Four non-helical or non-collagenous (NC) domains interrupt three helical/collagenous (COL) domains. COL 1 and COL 2 are involved in the interaction with collagen II fibrils via covalent cross-links (van der Rest and Mayne, 1988). The NC3 domain acts as a hinge, such that the COL 3 and NC4 domains extent outwards from the fibril surface. The NC3 domain of the α 2 (IX) chain contains a five amino acid insertion which provides an attachment site for a glycosaminoglycan side chain. The size of the side chain is variable. It is absent or small in cartilage and very large in avian vitreous humor (Brewton *et al.*, 1991). The N-terminal NC4 domain has an estimated isoelectric point of about 10, and is therefore thought to interact with the acid proteoglycans found in the cartilage matrix (Vasios *et al.*, 1988). However, the absence of an NC4 domain in avian vitreous humor suggests that an alternative function of collagen IX may be the attachment of the large glycosaminoglycan side chain to collagen II fibrils.

In cartilage, the N-terminal NC4 domain of the α 1(IX) chain is 243 residues long. However, in the primary stroma of the chick cornea this domain is only a few amino acid residues long (Svoboda *et al.*, 1988). This is due to the use of two different transcription start sites in the α 1(IX) gene (Nishimura *et al.*, 1989). An alternative promoter and first exon are located about 20 kilobase pairs downstream from exon 1 in an intronic sequence of the α 1(IX) gene (between exons 6 and 7). This alternative promoter and first exon have also been found in the mouse and human α1(IX) gene (Muragaki *et al.*, 1990).

Collagen XII is a homotrimer, [α 1 (XII)]3, containing two COL domains and one large N-terminal NC3 domain (Dublet *et al.*, 1989). Collagen XII interacts with collagen I fibrils in tendons and ligaments possibly via the COL 1 domain.

Collagen XIV is the latest FACIT to be discovered. It is a homotrimeric molecule found in skin and tendon (Gordon *et al.*, 1991). Characterisation of the molecule at the cDNA and protein levels indicates that it is similar to but clearly distinct from collagen XII.

1.3.3.3 Short-chain Collagens.

Molecules of collagens VIII and X are half the length of the fibrillar collagens and are therefore referred to as the short-chain collagens. As with all non-fibrillar collagens, their triple helices are interrupted by several short non-helical domains.

Collagen X is synthesised by hypertrophic chondrocytes during the process of endochondral bone formation (Schmid and Linsenmayer, 1987). The molecule is a homotrimer, [α 1 (X)]3, with three domains: (a) a short non-helical region at the N-terminus, (b) the main, interrupted, triple helical region, and (c) a globular C-terminus. Within the hypertrophic cartilage, collagen X is found as pericellular mats of filamentous material which surround the hypertrophic chondrocytes, and some is found along the surface of collagen II fibrils (Schmid and Linsenmayer, 1990; Poole and Pidoux, 1989). The structure of collagen VIII predicted from cDNA analysis is similar to that of collagen X. The non-helical N-terminal domain of collagen VIII is, however, considerably larger than that of collagen X, and a portion of the C-terminal globular domain shows some differences (Yamaguchi *et al.*, 1989). Collagen VIII was first isolated from endothelial cell cultures by Sage and Bornstein (1987) and is a component of the basement membrane (Descemet's membrane) separating corneal endothelial cells from the corneal stroma where it appears as a hexagonal network (Sawada *et al.*, 1990). Two chains have been characterised from

Descemet's membrane, α 1 (VIII) and α 2 (VIII). The stoichiometric arrangement of the two chains remains, however, to be established.

1.3.3.4 Collagen VI.

Collagen VI, which is found in most connective tissues, is a heterotrimer made up of three different chains: α 1 (VI), α 2(VI) and α 3 (VI) (Timpl and Engel, 1987). An unusual feature is the relatively short (105 nm) central triple helix which accounts for less than half of the total mass of the protein. The triple helix is further characterised by two non-helical interruptions and 11 Arg-Gly-Asp repeats throughout the triple-helix. Such sequences have been shown to have a role in the interaction of matrix proteins, in particular fibronectin, with integrin cell receptors (Sekiguchi, 1991). The non-helical N- and C-terminal domains are characterised by the presence of repeats showing significant similarities to the A domains of von Willebrand factor (van der Rest and Garrone, 1991).

The mode of molecular aggregation of collagen VI has been studied using rotary shadowing and electron microscopy of native and partially denatured or digested molecules (Timpl and Engel, 1987). Dimers are first formed by a staggered anti-parallel aligment of monomers with a 75 nm overlap in their triple-helical domains. Tetramers are then formed by the lateral association of dimers with their ends in register.

1.3.3.5 Collagen VII.

Collagen VII is found associated with the basement membrane of certain epithelial cell layers (e.g. at the dermal-epidermal junction in skin) where it forms anchoring fibrils linking the basement membrane to anchoring plaques found within the underlying matrix (Burgeson, 1987). It is thought to be a homotrimer consisting of a large, approximately 420 nm long, interrupted triple-helix and two non-helical domains, one at each end of the triple-helix. The N-terminal domain consists of three 50 nm long arms each terminated by a small globular domain. The C-terminal domain consists of a small globular region which is thought to be proteolytically removed during molecular assembly. Molecules first assemble into anti-parallel dimers with an overlap of 60 nm at the C-terminal end. The region of overlap is stabilised by disulphide bonds. The dimers then aggregate in register to form the anchoring fibril. The anchoring fibrils form a bridge between the basement membrane and the anchoring plaques probably via binding of the large globular N-terminal domain to collagen IV found both in basement membrane and in the anchoring plaques (van der Rest and Garrone, 1991). The resulting network entraps other matrix

components such as collagen I and collagen III fibrils, thus forming a firm connection between the epithelium and stromal matrix.

1.4 Collagen and the Lung.

1.4.1 Amounts and Types of Collagen.

It has been estimated that collagen comprises between 10-20% of the dry weight of the adult human lung and between 60-65% of lung connective tissue (Rennard and Crystal, 1982). Collagens I and III account for approximately 90% of lung parenchymal collagen in a ratio of 2 : 1 (Seyer *et al.*, 1976; Rennard and Crystal, 1982; Kirk *et al.*, 1984a). Immunofluorescence studies with type-specific antibodies have shown that collagens I and III are found throughout the interstitium of the alveolar structures (Madri and Furthmayr, 1980; Raghu *et al.*, 1985). Collagens I and III are also present in the connective tissue sheaths that surround the tracheobronchial tree and are found in all regions of the pulmonary arteries and veins as well as in the visceral pleura (Rennard and Crystal, 1982).

Collagen II in lung is confined to the cartilage of the trachea and large bronchi (Bradley *et al.*, 1974).

Collagen IV is the major component of basement membranes and therefore has been localised to the epithelial and capillary basement membranes of the alveolar structures where it constitutes approximately 5% of parenchymal collagen (Rennard *et al.*, 1980a; Madri and Furthmayr, 1980). It is also found in the endothelial basement membranes of the pulmonary arteries and veins and the epithelial basement membranes of the tracheobronchial tree.

Collagen V is also present in basement membranes. In the alveolar structures it is not clear whether it is limited to the epithelial and endothelial basement membranes or whether it is present in the interstitial matrix as well (Madri and Furthmayr, 1979; 1980). Collagen V is thought to constitute approximately 5% of parenchymal lung collagen (Rennard *et al.*, 1980a).

It seems likely that some of the minor collagens which are associated with certain fibrillar collagens in other areas of the body will be found in the lung. Thus, collagen VI may be found co-distributing with collagens I and III in the pulmonary vasculature and interstitium and collagens IX, X and XI may be associated with collagen II in cartilage (Laurent, 1986).

The collagen content (amount/dry weight) of the tracheobronchial tree and pulmonary vasculature is greater than that in the parenchyma. However, as the total mass of the parenchyma is much larger, the bulk of the collagen is found in the alveolar structures (Rennard and Crystal, 1982).

The data available on the collagen content of lung in relation to age is conflicting. Pierce and Hocott (1960), and Wright *et al.*, (1960) found no significant correlation between right middle lobe collagen or total lung collagen, respectively, and age. Briscoe *et al.*, (1959) showed an increase in parenchymal collagen, as a percentage of dry weight, with increasing age, while Johnson and Andrews (1970) showed a decrease in parenchymal collagen, as a percentage of total ("crude") parenchymal connective tissue, with increasing age. These disagreements seem to be due to sampling techniques and to the way the data is expressed. Andreotti *et al* (1983) found a decrease in collagen with age when the data were expressed, more appropriately, per unit volume of lung parenchyma after inflation at a standard pressure of 25 cm H_20 followed by fixation with formalin. Andreotti *et al.* (1983) found no correlation between collagen content and age when collagen was expressed per gram dry weight of tissue, a method which does not take into account possible changes from normal in the amount of alveolar wall per unit volume.

1.4.2 Turnover.

During normal growth and development, as well as repair of tissues, it is important that the process of collagen synthesis be counterbalanced by collagen degradation. The major pathway of degradation involves a group of enzymes known as the collagenases (Harris *et al.*, 1984; Jeffrey, 1986). These are neutral metallo-proteases requiring both Ca^{2+} and Zn^{2+} for activity. The best characterised collagenases are capable of degrading collagens I, II and III. They attack the collagen molecule at one specific site in the triple helix to produce a N-terminal three-quarter fragment and a C-terminal one-quarter fragment. At body temperature the fragments denature to random coils and are further degraded by other proteases. The fragments may also be taken up by cells by phagocytosis and be further degraded within lysosomes (Etherington, 1980). The cathespins B and N, found within lysosomes, have been shown to have collagenolytic activity.

Most studies of collagen turnover have utilised radiotracer techniques where the incorporation of radiolabelled proline into collagen is followed. Synthesis is determined from the specific radioactivity of proline and hydroxyproline in collagen

extracted at various time points. Similarly, degradation can be determined from the specific radioactivity of hydroxyproline in the tissue-free pool.

Using these techniques it has been shown that lung collagen in rats and rabbits is being synthesised at a rate of about 9-10% per day (i.e., an amount of collagen equivalent to 9-10% of the total pool), and that a significant proportion (up to a third) of newly synthesised collagen (procollagen) is degraded rapidly after its production, probably intracellularly (Laurent, 1982; McAnulty and Laurent, 1987). Because radiotracer studies are limited to a few hours duration, the measured rates of collagen turnover reflect only the early phases of the molecular assembly of collagen and not the turnover of mature cross-linked collagen. Cross-linking and maturation of collagen has been shown to take days and even weeks for completion (Pinto and Bentley, 1974).

It seems likely that turnover of mature cross-linked collagen is most relevant in pathophysiological processes in the lung. However, Laurent and McAnulty (1983) demonstrated both an increased synthesis and decreased degradation of collagen in the early stages of fibrosis in rabbits caused by the intratracheal instillation of bleomycin.

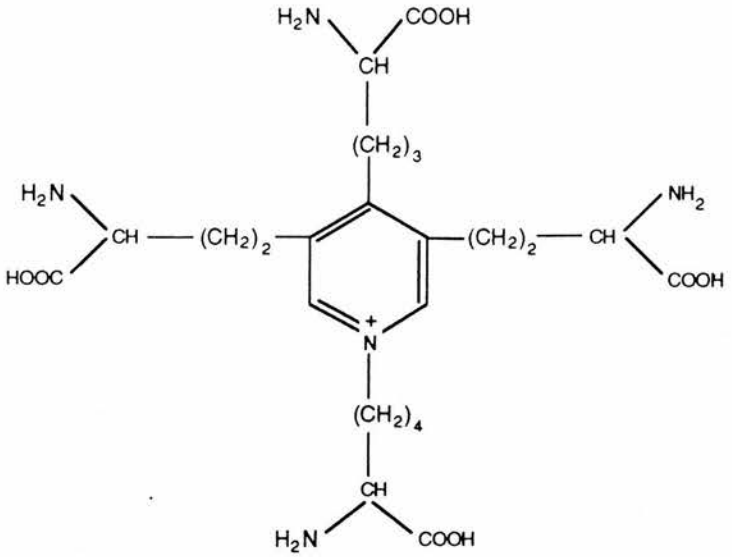
1.5 Elastin.

Elastin is a major component of the connective tissue of blood vessels and the lung. It is also present in elastic cartilage, skin, ligaments and the uterine cervix. Elastin exhibits an intrinsic elasticity that gives the tissues where it is present an extensibility and subsequent recoil dependent only on the application of some physical force.

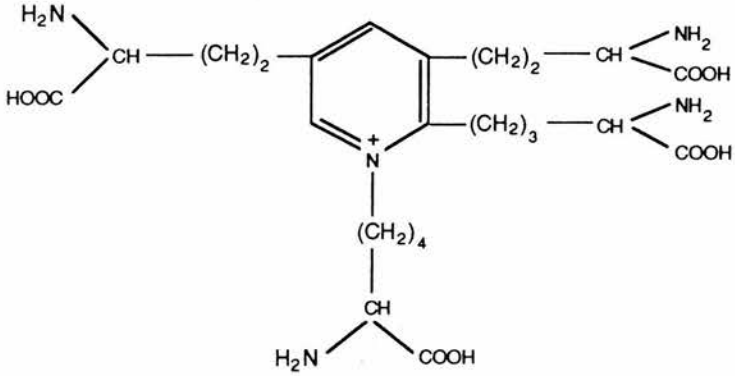
The elastic fibre is composed of two chemically and morphologically distinct components. An amorphous component, elastin, is the major fraction, comprising 90% of the mature fibre. Unlike collagen, elastin lacks any repeating pattern when seen under the electron microscope. The second, microfibrillar, component is a glycoprotein which appears as small fibrils 10 - 12 nm in diameter located at the periphery of the amorphous elastin. Evidence suggests that the microfibrils are secreted into the extracellular matrix before elastin synthesis and function as a nucleation site for elastin deposition (Ross *et al.*, 1977). As development proceeds, the fibrillar component becomes saturated with the elastin and as a result is seen only at the periphery of mature elastin fibres. Kadar and Robert (1975) demonstrated morphologically that removal of the elastin component resulted in the appearance of the underlying fibrillar component. Elastin synthesis has been documented in fibroblasts,

smooth muscle cells, chondrocytes, endothelial cells and, more recently, in lung mesothelial cells (Mecham *et al.*, 1981; Burke and Ross, 1979; Quintarelli *et al.*, 1979; Cantor *et al.*, 1980; Mecham *et al.*, 1983; Mandl *et al.*, 1986). The process is initiated by transcription from the elastin gene and data indicates that elastin synthesis is regulated by the transcription and availability of elastin messenger RNA (Burnett *et al.*, 1980). Translation of the messenger RNA produces the soluble precursor molecule, tropoelastin, first purified and partially characterised by Sandberg *et al.* (1969) from copper-deficient porcine aorta. Tropoelastin is secreted into the extracellular matrix as a protein with a molecular weight of 72,000 and with a similar amino acid composition to insoluble elastin except that it contains additional lysine residues. Recent evidence suggests that alternative splicing of a single elastin gene may give rise to distinct messenger RNA molecules (Indik *et al.*, 1987; Raju and Anwar, 1987; Yeh *et al.*, 1989). Translation of such elastin messenger RNA molecules would result in significant variation in amino acid sequence. In fact three tropoelastin isoforms have been identified in chick lung (Rich and Foster, 1987), and a variety of bovine tissues (Wrenn *et al.*, 1987; Parks *et al.*, 1988). The proportion of the bovine tropoelastin isoforms appear to vary according to the stage of development (Parks *et al.*, 1988).

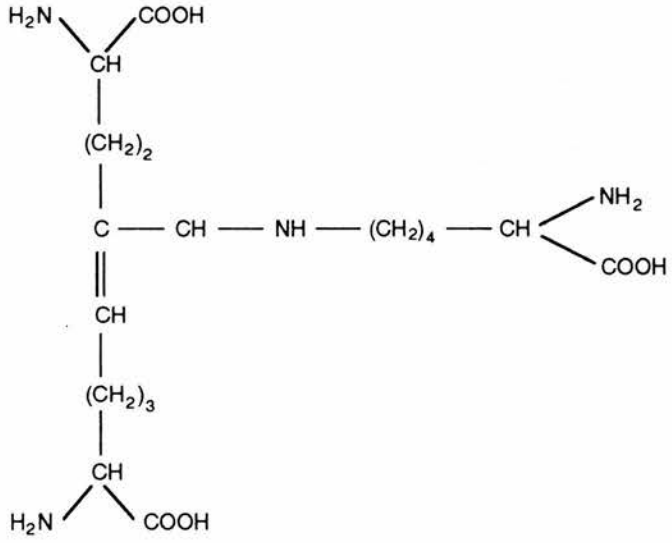
Elastin is cross-linked by the unique amino acids, desmosine and isodesmosine (Partridge *et al.*, 1963; Thomas *et al.*, 1963). Lysyl oxidase is the enzyme that initiates the biosynthesis of the cross-links by catalysing the oxidative deamination of the ε amino groups of all but five or six of the total 37 lysine residues to α-aminoadipic semi-aldehydes (Pinnell and Martin, 1968). These reactive residues (allysines) spontaneously condense to form various Schiff base and aldol condensation crosslinks. Within a few days most of the cross-links have isomerised into the stable quarternary pyridinium ring structures of desmosine and isodesmosine (Figure 1.3). Desmosine and isodesmosine are tetrafunctional but normally join only two chains. Sandberg and Davidson (1984) hypothesised that the amino acid sequence - Lys - Ala - Ala -Lys - of one molecule meets the amino acid sequence - Lys -Ala - Ala - Ala - Lys - of another molecule. Three Lys residues in these sequences are converted to allysine residues and the fourth provides the ring nitrogen of the pyridinium cross-link. It is not clear how the final cross-links form. However, coacervation may play a role by closely apposing the appropriate residues (Urry *et al.*, 1974). Two other cross-linking amino acids have also been found, lysinonorleucine, (Franzblau *et al.*, 1965) and merodesmosine (Starcher *et al.*, 1967; Figure 1.3). Both derive from lysine.



Desmosine



Isodesmosine



Merodesmosine

Figure 1.3 The structures of the elastin-specific cross-links desmosine, isodesmosine and merodesmosine.
Adapted from Mecham and Heuser (1991).

One of the main features of elastin-containing tissues is their ability to be deformed to large extensions by relatively small forces and then return to their original dimensions. Elastin can be extended by 100-150% before it breaks, compared to fibrillar collagen which breaks at extensions of about 5 - 8%. The precise physicochemical properties that account for elastin's rubber-like characteristics have not been fully characterised. Thermodynamic studies of purified elastin led Hoeve and Flory (1974) to propose that elastin behaves as a classical "rubber" consisting of cross-linked chains randomly oriented. According to the model, an unstretched rubber network is in a state of maximum entropy (i.e. maximum disorder), and an external force that acts to extend the network will increase the order of the network (i.e. decrease its entropy) by imposing a degree of preferred orientation in the direction of extension on each of the random chains in the network. When the external force is removed, elastic recoil occurs because the ordered chains return to their initial, disordered state according to the second law of thermodynamics which states that systems proceed spontaneously to a state of maximum entropy. Nuclear magnetic resonance (NMR) studies on elastin indicate that the backbone chain of the elastin monomer is highly mobile (Torchia and Piez, 1973; Fleming *et al.*, 1980).

Although the dominant molecular pattern of elastin is that of a kinetically free, random-coil network there are some features of elastin structure that indicate regions of local order. Circular dichroism has indicated the presence of helical secondary structure associated with the alanine-rich sequences in the crosslink regions (Foster *et al.*, 1976). Several hydrophobic repeat sequences are also found in elastin, including the pentapeptide Pro - Gly- Val- Gly - Val and a hexapeptide Pro - Gly - Val - Gly - Val - Ala. It has been shown that both sequences contain a β turn and, consequently, when repeated several times on a helical axis form a β spiral (Urry, 1983). These areas of secondary structure could serve to restrict movement around chemical bonds and, therefore, the kinetic mobility assumed for an ideal rubber may not hold for all areas of elastin.

Elastin is an extremely hydrophobic protein with approximately 70% of its amino acid side chains being nonpolar. This property has led several workers to propose alternative models of elastin structure which account for its elasticity (Weis-Fogh and Andersen, 1970; Gray *et al.*, 1973; Urry *et al.*, 1974). These models stress the importance of water-protein interactions in the mechanism of elastic recoil. The authors state that hydrophobic interactions are disrupted when nonpolar regions of the molecule are exposed to water when elastin is stretched. Elastic recoil occurs due to the reaggregation of the nonpolar groups with the expulsion of the absorbed water. Like the "rubber" model the elastic energy is stored as

a decrease in entropy though in this model the entropy is largely associated with the water. Further thermodynamic studies made by Gosline (1978) support the idea that water-protein interactions contribute to the elastic mechanism but the results are in no way at variance with the "rubber" model. The actual mechanism is probably a dynamic blend of these two extremes.

1.6 Elastin and the Lung.

The elasticity of the lung is determined most importantly by elastin. Elastic fibres are ubiquitous in the lung, and as with other organs these fibres are closely associated with collagen and proteoglycans. Morphologic studies have demonstrated elastic fibres in all the major lung structures, including airways, blood vessels, parenchyma, and pleura (Starcher, 1986). In the larger pulmonary arteries and airways elastic fibres are present both as loops and as longitudinal bundles. In the vessels fibres are more prominent in the media but they are also evident in the adventitia and intima. The fibres are also seen in the submucosal areas of the airways. In the respiratory bronchioles, elastic fibres become more distinct distally.

Light microscopic observations have shown that elastic and collagen fibres assume an integral fibre network which forms the architectural skeleton of the alveolar structure. Elastic and collagen fibres encircle respiratory bronchioles and alveolar ducts in a helical fashion. They then completely encircle the alveolar mouths before appearing as a fine mesh in the alveolar walls (Pierce and Ebert, 1965; Young *et al.,* 1980). Using electron micrographs, Mercer and Crapo (1990) produced reconstructions of elastic and collagen fibres in order to determine their spatial arrangement within the acinar unit. These authors found that in normal human lungs the highest concentrations of collagen and elastic fibres were found in the alveolar tissue, where they surrounded the alveolar ducts. In this region the collagen-to-elastic fibre ratio was 1.0. Further out from the alveolar ducts, in the alveolar walls, the collagen-to-elastic fibre ratio was 1.5-2.0. They also found that the elastic fibres encircling the alveolar mouths occurred in two configurations relative to the adjacent collagen fibres. In one configuration, the elastic fibres were in intimate contact with the adjacent collagen fibres and were seen to be interwoven with numerous collagen fibrils of the collagen fibre, indicating some sort of mechanical interconnection. In the second configuration, elastic fibres were in a tight band-like configuration and were spacially separated from the adjacent collagen fibres. Collagen fibrils of the alveolar duct wall were found to have a wave-like arrangement and meandered about one another with no apparent order or uniform pattern.

The close association of collagen and elastic fibres does not seem to support the theory that axial elongation of elastic fibres accounts for the elasticity of the lung. While elastin has a large range of extensibility and exhibits ideal elastic behaviour, collagen has a high tensile strength and a short range of extensibility. Elastic fibres, therefore, may be unable to undergo axial elongation owing to the inelasticity of collagen. The spiral arrangement of closely associated elastic and collagen fibres around the alveolar ducts led Pierce and Ebert (1965) to propose an alternative model. They proposed that the lung expands by unfolding its structure with the extension of the ductal helices. This uncoiling of the helices produces torsion or twisting in the tissue fibres leading to elastic recoil as the fibres react against this deformation. The Setnikar and Mead model (for review see Snider and Karlinksy, 1977), on the other hand, proposes that elastic and collagen fibres operate in parallel and independently of each other. The model, based on the observations made by Orsós (1907) and von Hayek (1960) that the collagen fibres of human lungs appear as wavy bundles, assumes the collagen fibres to be spirally coiled or serpentine at resting lung volume. At low lung volumes the elastic fibres are extensible. As lung volume increases the coiled collagen fibres straighten, resulting in a falling compliance of the combined networks and greatly increased stiffness of the lungs at maximal lung volume. The collagen network is, therefore, believed to limit total pulmonary distention. *In vitro* and *in vivo* studies, using collagenase or elastase for selective disruption of connective tissue components, support the theory of parallel functioning of elastic and collagen fibres (Snider and Karlinsky, 1977). Most recently, Mercer and Crapo (1990) found that collagen fibrils of alveolar wall collagen fibres have a wavelike arrangement, in keeping with the model, and may be extended by as much as 16% before they become straight and limit further extension.

Elastin has been estimated to constitute approximately 18% of dry weight of the lung connective tissue (Pierce *et al.*, 1961). Dissection of the lung into pleura, bronchioles, large blood vessels, and parenchyma has shown that the highest amount of elastin is found in parenchyma (16-40% dry weight of connective tissue; Pierce and Ebert, 1965).

Many authors have reported an increase in lung elastin with age. Pierce and Hocott (1960) found an increase in the elastin content of the right middle lobe with increasing age. Wright *et al.* (1960) also found an increase in total lung elastin with age. Briscoe and Loring (1958) and Johnson and Andrews (1970) showed an increase in parenchymal elastin with increasing age when expressed as a percentage of dry weight of tissue or as a percentage of total ("crude") connective tissue, respectively. Pierce and Ebert (1965) and John and Thomas (1972), however,

could demonstrate an age related increase in the elastin content only in the pleura, with no change in the parenchyma. As with the studies on the collagen content of the lung with increasing age, the disagreements seem to be due to sampling technique and to the way the data is expressed. Andreotti *et al.* (1983) reported an increase in parenchymal elastin with age when expressed in milligrams per gram of dried parenchyma, but could find no correlation with age when elastin content was expressed per unit volume of parenchyma after inflation at standard pressure of 25 cm H_2O followed by fixation with formalin.

Physiological turnover of insoluble elastin appears to be very slow. Under normal conditions the mature lung parenchymal elastin is metabolically stable over the human life span (Shapiro *et al.*, 1991).

1.7 The Collagen and Elastin Content of the Lung in Emphysema.

The earliest studies of the collagen and elastin content of normal and emphysematous lungs were made by Wright *et al.* (1960) and Pierce *et al.* (1961). Whole lung measurements did not show any alteration in the content of collagen or elastin in severe pulmonary emphysema as compared to a series of control lungs. A study of individual tissue blocks also showed no relationship between collagen and elastin content and the severity of emphysema (Pierce *et al.*, 1961). Pecora *et al.* (1967) refined the sampling technique and obtained 5 gram pieces of lung tissue from multiple sites within a lung to get a more representative picture. They found that the mean collagen content per 100 grams wet weight of tissue in the emphysematous group was greater than, and the elastin values were about the same as, that of the control group. Importantly, the data of Pecora *et al.* (1967) were more representative of parenchymal tissue, since most of the samples were obtained from peripheral areas that are relatively low in bronchial or brochiolar tissue. Total lung collagen content of the emphysematous lungs was greater than, and the elastin content was less than, that of the control group. Chrzanowski *et al.* (1980) measured the elastin specific cross-linking amino acids desmosine and isodesmosine in 1 gram aliquots of lung parenchyma, after removal of pleura, large airways and blood vessels, in normal lungs and in lungs with panacinar emphysema associated with abnormal alpha 1-Pi phenotypes. Elastin proportions were expressed as a percentage of parenchymal crude connective tissue. The results showed significantly reduced proportions of lung elastin in pulmonary emphysema except in patients treated with glucocorticoids which may decrease collagen synthesis and, therefore, increase the relative proportions of elastin.

Biochemical measurements have also been made on animal models of emphysema (for reviews see Karlinsky and Snider, 1978; and Snider *et al.*, 1986). In general, total lung elastin has been shown to be significantly reduced immediately after intratracheally instilled elastase but then returns to normal after several weeks. *In vivo* radiolabelling techniques have confirmed biosynthesis of lung elastin after its degradation. Total lung collagen changes little after elastase treatment. However, *in vivo* radiolabelling with ^{14}C-proline has revealed an increase in collagen biosynthesis following elastase treatment (Yu and Keller, 1978; Kobrle *et al.*, 1982; Karlinsky *et al.*, 1983). These studies indicate that damage and repair of collagen are a part of elastase-induced emphysema. However, the proportion of total lung collagen damaged must be very small, since highly sensitive radiolabelling techniques must be used to show it.

Belton *et al.* (1977) showed ultrastructural abnormalities of the collagen fibrils within the alveolar walls in areas of emphysema. The collagen fibres lost the parallel orientation observed in normal regions and were randomised in alveolar walls adjacent to foci having alveolar enlargement. Collagen fibrils in regions of severe emphysema appeared enlarged and swollen. No change in the quantity of elastic fibres could be seen when nonemphysematous areas were compared with areas of mild or severe emphysema. However, there did appear to be an alteration in the distribution of elastic fibres. Aggregations of dense elastic fibres were frequently clumped within the free edges of alveolar walls. Fukuda *et al.* (1989) demonstrated abnormal elastic fibres by electron microscopy and by light and electron microscopic immunohistochemistry for elastin in a patient with panacinar emphysema due to alpha 1-antitrypsin deficiency and in three patients with centriacinar emphysema related to anthracosis. Four types of abnormal elastic fibres were found: (1) finely disrupted fibres; (2) fibres with vacuolar changes and deposits of electron-dense granular material ; (3) accumulations of small, rounded amorphous components of elastic fibres near bundles of micro-fibrils; and (4) large, confluent masses consisting mainly of aggregates of irregularly and compactly arranged, small-sized amorphous components. The amorphous components stained evenly with anti-elastin antibody. Finely disrupted fibres were frequently found in the patient with panacinar emphysema and were presumed to have been damaged by elastase. The other three types of elastic fibres were frequently found in the patients with centriacinar emphysema. They suggested that: (1) the vacuoles and electron-dense deposits in elastic fibres probably represented damage to elastic fibres; (2) the small round amorphous components in elastic fibres might be formed from abnormal elastogenesis; and (3) the large, confluent elastic masses were formed by the aggregation of elastic fibres in areas of coalescence of alveolar walls undergoing structural remodelling.

Nagai and Thurlbeck (1991) performed dissecting light and scanning electron microscopy on 14 lungs with mild centrilobular emphysema, two of which also had mild panacinar emphysema. The lungs were surgically resected, mainly for cancer. The walls of centrilobular emphysematous spaces were either thick or thin. The former were less frequent but contained considerable fibrous tissue. The thin walls contained focal areas of fibrosis. No fibrosis was seen in the walls of panacinar emphysematous spaces. These observations seem to challenge the definition of emphysema and raises the question, what is meant by excess fibrosis? They concluded that some sort of quantitative study should be made of the amount of collagen in emphysematous spaces.

1.8 Aims.

Evidence from animal models of emphysema and more recently from microscopic observations of emphysema in humans, suggests that both elastin and collagen are affected during the pathogenesis of emphysema. It would appear that as well as degradation there is resynthesis, or repair, or even redistribution of elastin and collagen.

Previous studies on the collagen and/or elastin content of lung in relation to emphysema can be critisised either because whole lungs were used or the reference terms were inappropriate. The use of whole lungs (Wright *et al.*, 1960; Pierce *et al.*, 1961) fails to recognise the heterogeneity of the lung structure with respect to collagen and elastin content (Pierce and Ebert, 1965). Since emphysematous lesions are limited to the acinar unit of the lung, measurements on the whole lung or on one of its lobes may fail to show changes in lung parenchyma. Both studies cited above could not show a difference in the collagen and elastin content of whole emphysematous lungs compared to non-emphysematous lungs.

The second criticism relates to the reference terms used for the expression of the data. Previous studies have failed to take into account changes in the amount of alveolar wall in emphysema. Thus Pecora *et al.* (1967) expressed collagen and elastin per 100 grams wet weight of parenchyma. Johnson and Andrews (1970), Chrzanowski *et al.* (1980), and Tarján *et al.* (1989) expressed their data as a percentage of total ("crude") connective tissue in samples of lung parenchyma. Andreotti *et al.* (1983) stated "in the quantitative analysis of connective tissue, data should be expressed referring to the spatial unit of measurement which is specific for the structure under examination". Therefore, when studying connective tissue in

lung, data should be expressed with reference to a unit volume of inflated lung parenchyma.

When expressed as micrograms per cubic centimeter of inflated lung parenchyma, Andreotti *et al.* (1983) found that collagen content decreased with age while elastin content did not show any significant correlation with age. It has been estimated morphometrically that between one-third (Thurlbeck, 1967b) and one-quarter (Gillooly and Lamb, 1993a) of the total alveolar walls is lost between the ages of 20-80 years. Clearly interpretation of collagen and elastin content of alveolar walls in relation to emphysema must take into consideration the aforementioned findings.

The aims of the present study were to make measurements of collagen and elastin on the alveolated portions of lungs, after removal of pleura, large airways and blood vessels and to correlate these measurements with those of airspace size, with particular reference to microscopic emphysema.

The specific aims were two-fold:

(1) to quantitate total collagen and elastin in the alveolar walls of emphysematous and non-emphysematous lungs;

(2) to quantitate the different genetic types of collagen in the alveolar walls of emphysematous and non-emphysematous lungs.

CHAPTER 2. MATERIALS AND METHODS

2.1 Tissue Sampling and Morphometry.

2.1.1 Fresh Lung Samples.

Whole lungs or lobes were obtained from patients who had undergone surgery for the removal of peripheral tumours at the City Hospital, Edinburgh. All patients had a history of smoking, were aged between 55-85 years and showed no evidence of macroscopic emphysema.

An additional two lungs were obtained at autopsy from a 75 year old smoker and an 85 year old non-smoker, neither of whom showed any signs of macroscopic emphysema.

2.1.1.1 Agarose Inflation and Sampling of Resected Lung Samples.

In order to express the biochemical data in relation to lung structure, a technique was developed which allowed collagen and elastin content to be measured on known volumes of fresh, inflated lung. The technique of agarose inflation was developed because the standard procedure of inflation by intrabronchial perfusion with buffered formalin (Wentworth, 1950) was thought to be unsuitable due to the reaction of formaldehyde with the amino groups of lysine (Kadler and Chapman, 1988) and therefore possible interference with the determination of the elastin specific cross-linking amino acids desmosine and isodesmosine, although it was recognised that the amino groups of the lysine residues are not free in the peptide-bound cross-links.

Following removal, lung tissue was immediately placed on ice and sent to the Department of Pathology, University of Edinburgh, where it was further processed and analysed morphometrically by Dr. M.R. Lang. A random zone of lung, approximately 100mm x 50mm x 50mm and well away from the tumour, was transpleurally inflated by injecting 150-200 ml of a 2% agarose solution heated to 30°C. The zone was considered inflated when the surface of the pleura became firm and smooth in appearance. The tissue was kept on ice for a further 10 minutes until the agarose had solidified within the airspaces, effectively maintaining alveolar architecture. The inflated zone was then dissected free from the surrounding lung, trimmed of pleura and stored at 4°C for a further 20 minutes. After placing the zone on a purpose built template, slices approximately 20mm x 20mm x 2mm were taken with a surgical skin graft knife blade. The position of each slice within the zone was recorded and alternate slices were stored in 10% buffered formalin for histology and subsequent morphometry, and 70% ethanol for collagen and elastin determinations.

The slices for biochemical measurements were further subdivided and their co-ordinates from within the larger slice recorded and their exact dimensions determined with calipers. All samples were then examined microscopically and those containing large amounts of broncho-vascular material were discarded. From the nine resected lungs obtained, a total of 102 samples were prepared for biochemical analysis. Figure 2.1 shows a schematic representation of the sample preparation procedure.

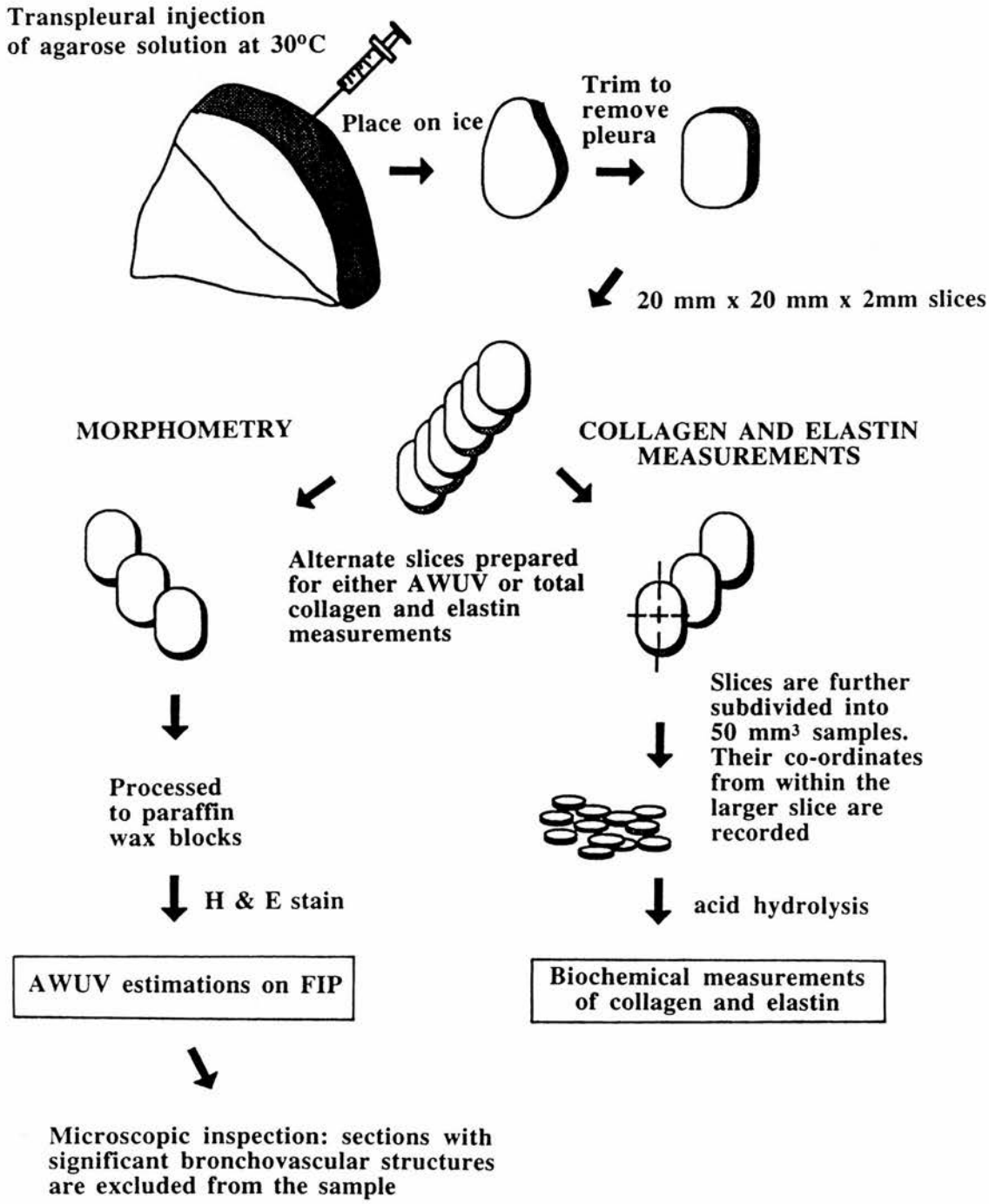


Figure 2.1. Schematic representation of agarose inflation and sampling of resected lung samples for morphometry and biochemical analysis.

2.1.1.2 Sampling of Post Mortem Lungs.

In order to study regional variation in airspace size, and collagen and elastin within a lung, the two whole autopsy lungs were examined. Three zones (upper, middle and lower) within both upper and lower lobes from the right lung were prepared as above. The data obtained from regions within each lobe and between upper and lower lobes were analysed using one-way analysis of variance (ANOVA) on the Apple MacIntosh version of the program Minitab.

2.1.2 Formalin Inflated Lung Samples.

Samples were also obtained from a store of formalin inflated lungs kept in the Department of Pathology, University of Edinburgh. Whole lungs were obtained at autopsy (one lung from each subject), and lobes were obtained from patients undergoing surgery for the removal of peripheral tumours. These lungs and lobes represented 14 lifelong non-smokers (age range 22 - 82 years) and 29 smokers (age range 43 - 82 years). The midsaggital slice of each lobe was examined by an experienced pathologist (Dr. D. Lamb, Department of Pathology, University of Edinburgh) in order to determine the type of macroscopic emphysema when present. Macroscopic emphysema was considered present if airspace size was greater than 1 mm in diameter (Gould *et al.*, 1988). The smoking group thus comprised 11 cases with centriacinar emphysema, nine cases with panacinar emphysema, five cases with a mixture of centriacinar and panacinar emphysema, and four cases with no macroscopic emphysema.

2.1.3 Alpha 1-Protease (α1-Pi) Deficient Lungs.

Lungs from six subjects were obtained at autopsy. Of these lungs, three were from subjects with α1-Pi deficiency, kindly supplied by the Heart and Lung Unit, Freeman Hospital, Newcastle-upon-Tyne. The genotype and smoking histories of these subjects were, however, not available. The remaining lungs were from two heavy smokers and one non-smoker.

2.1.4 Morphometry.

2.1.4.1 Preparation of Agarose Inflated Tissue.

Formalin fixed slices of agarose inflated lung were dehydrated through a graded ethanol series and embedded in paraffin. From each slice histological sections were

cut at 4 μm on a rotary microtome, and floated onto microscope slides. The sections were de-waxed in xylene and stained by the haematoxylin and eosin method of Culling (1963). Alveolar wall surface area per unit volume of lung (AWUV) was determined for each 1 mm^2 field in an 121mm^2 region within each section by Dr. M.R. Lang (Department of Pathology, University of Edinburgh) using a Fast Interval Processor (FIP; Tucker and Shippey, 1983; Aherne and Dunnill, 1982).

The dimensions of paraffin embedded and agarose inflated fresh tissue differed slightly due to shrinkage during processing to paraffin. It was therefore necessary to calculate a shrinkage factor for each section and adjust the morphometric data accordingly. To do this, tissue slice areas were measured before and after processing. The ratio of these measurements was used to calculate the area shrinkage, and the square root of this ratio gave the linear shrinkage. As the dimensions of AWUV are mm^{-1} (ie. mm^2/mm^3), all AWUV results were corrected by the linear factor (Gillooly *et al.*, 1991).

2.1.4.2 Preparation of Formalin Inflated Tissue.

Lungs or lobes were floated in 10% buffered formalin and inflated by intrabronchial perfusion with 10% buffered formalin at a pressure of 25cm H_2O from an elevated reservoir until the pleural surface became firm and smooth. Mean case AWUV was estimated on a FIP by Dr. M. Gillooly (Department of Pathology, University of Edinburgh) from six random 20mm x 20mm x 5mm blocks taken from the midsaggital slice of each lobe (Gillooly and Lamb, 1993a). The blocks were embedded in glycol methacrylate resin and from each block 3 μm sections were cut. The sections were then stained by the haematoxylin and eosin method of Culling (1963). AWUV was determined for each 1 mm^2 field in an 121mm^2 region within each section.

A further 20mm x 20mm x 5mm block was taken from the midsaggital slice of each lung for further AWUV measurements and for collagen determinations. In the emphysematous lungs, blocks were taken from areas free of macroscopically visible emphysematous lesions as it was not possible to determine AWUV values in areas of macroscopic emphysema. Furthermore, it was felt that sampling areas remote from gross lesions would be more indicative of the early stages of the disease process. A technique was developed by Dr. M.R. Lang (Department of Pathology, University of Edinburgh) which allowed the determination of both AWUV and collagen to be made on adjacent histological sections. Blocks of tissue were removed from the formalin fixative and placed on an absorbent tissue. Gentle pressure was then

applied in order to expel the formalin from the blocks. The blocks were then immersed in an Optimal Cutting Temperature compound (Tissue-Tek; Bayer Diagnostics, England) for 15 min until the airspaces were fully penetrated. Tissue-Tek maintains airspace inflation and block rigidity during histological processing. The tissue was then frozen in liquid nitrogen and four 20mm x 20mm x 25μm serial sections (total volume 40mm^3) were cut on a cryostat for collagen determination. An adjacent 3 μm section was cut for AWUV determination. Figure 2.2 shows a schematic representation of the sample preparation technique.

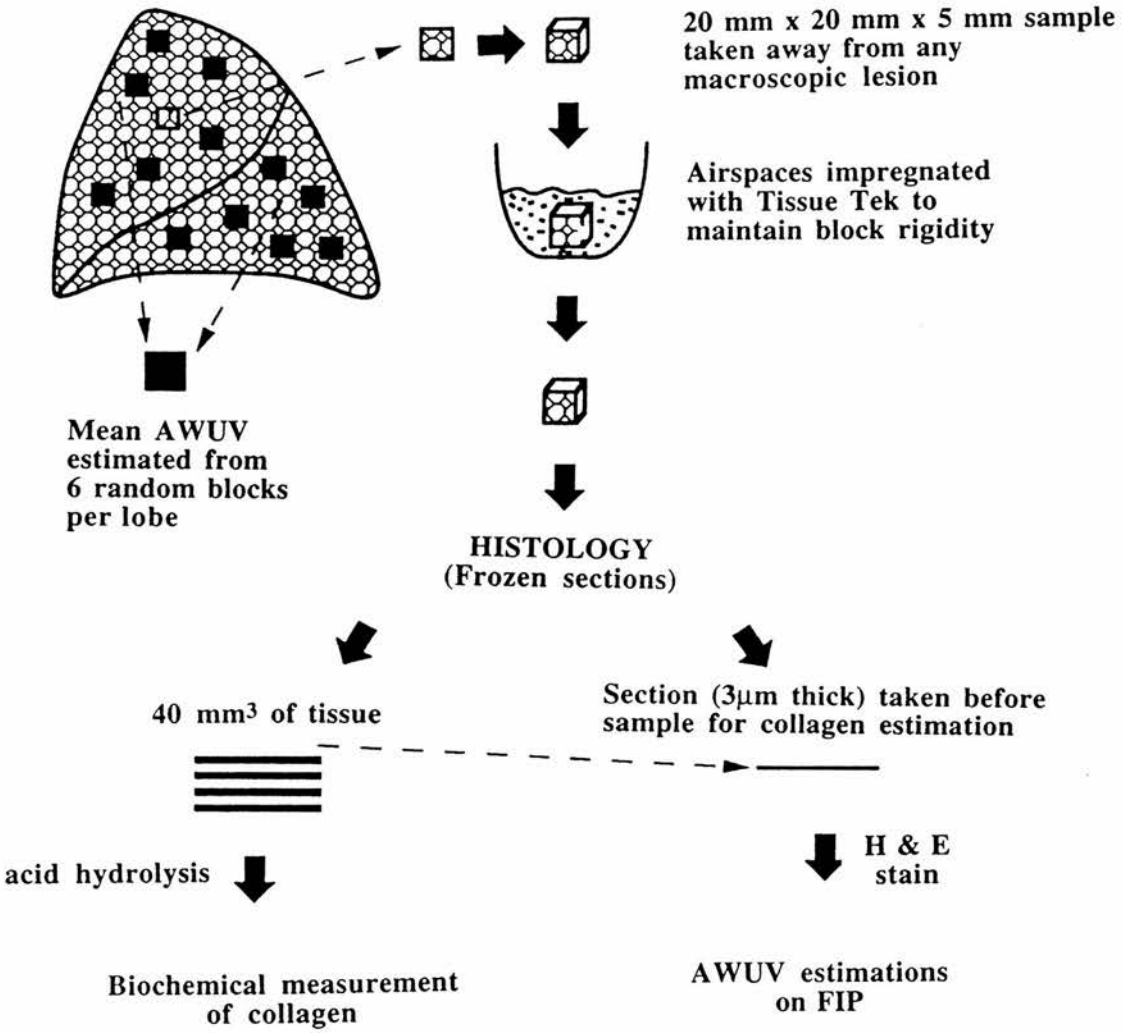


Figure 2.2. Schematic representation of the sampling procedure of formalin inflated lungs for morphometry and biochemical analysis.

2.1.4.3 Preparation of α1-Pi Deficient Lungs.

One lobe from each lung was inflated with 10% buffered formalin as described in section 2.1.4.2. Between five and eight random 20mm x 20mm x 5mm blocks were then prepared using the technique developed by Dr. M.R. Lang (Department of Pathology, University of Edinburgh) which allows the determination of both AWUV and collagen to be made on adjacent histological sections (section 2.1.4.2). Thus four 20mm x 20mm x 25μm serial sections (total volume $40mm^3$) were pooled for collagen determination and an adjacent 3μm section was prepared for AWUV determination.

The second lobe of each lung was inflated with 70% (v/v) ethanol at a standard pressure of 25cm H_2O in order to allow the determination of elastin to be made. Between 5 and 9 random 20mm x 20mm x 5mm blocks were then prepared using the technique developed by Dr. M.R. Lang (Department of Pathology, University of Edinburgh) and described in section 2.1.4.2. A total of fifteen 20mm x 20mm x 25μm serial sections (total volume $150mm^3$) were pooled for collagen and elastin determination and an adjacent 3μm section was prepared for AWUV determination.

The lungs from the two heavy smokers and from the non-smoker were prepared in exactly the same way.

2.1.4.4 AWUV Measurement by the Fast Interval Processor (FIP).

Emphysema has traditionally been assessed macroscopically by examining whole lung slices (Dunnill, 1962, 1970; Ryder *et al.*, 1969; Thurlbeck, 1967(a); Thurlbeck *et al.*, 1968, 1970). However, by the time emphysematous spaces are visible to the naked eye as much as 75% of the alveolar surface area may have been lost (Lamb, 1990). Since the interest of this study was early emphysema, a technique was used which allowed changes in the amount of alveolar wall to be assessed microscopically. All morphometric measurements were carried out by Dr. M.R. Lang and Dr. M. Gillooly (Department of Pathology, University of Edinburgh).

The FIP (Tucker and Shippey, 1983), is a rapid scanning device which has been adapted for use with lung tissue. It uses the technique of mean linear intercept (Lm; Dunnill, 1962, 1964; Duguid *et al.*, 1964; Thurlbeck, 1967a,b,c; Bignon *et al.*, 1969; Depierre *et al.*, 1972; Hasleton, 1972; Hansen and Ampaya, 1975), which has become the standard technique for measuring alveolar surface area on tissue sections. The technique involves counting the total number of intercepts made by a test line in a histological section of alveolar tissue. This figure is then used to calculate the average

distance between intercepts and from this alveolar wall surface area per unit volume (AWUV, expressed as mm^2/mm^3) can be derived (Aherne and Dunnill, 1982).

The FIP consists of a computer linked Nikon inverted microscope equipped with a motorised stage and a Fairchild CCD linear image sensor. The sensor consists of a stationary array of photosensitive units which recognise the optical density pattern of the specimen. Each histological section was scanned electronically in the y-axis by the sensor at 10 μm intervals. Sections were scanned mechanically in the x-axis by moving the stage in 1 μm steps. In total, an area of 121 mm^2 was scanned on each histological section. The image obtained from the scan consists of a grid of picture elements or "pixels". A user-defined threshold limit determines which pixels are recognised as tissue pixels and which are background pixels. A size filter then ensures that groups of thresholded pixels less than 6μm in diameter are ignored, thus eliminating cells, debris, and background "noise".

For ease of calculation, intercept totals for each 1 mm^2 area (from within the 121 mm^2 field) were stored by the computer. Each electronic scan creates a "test-line" 1 mm in length for each 1 mm^2 area, and, as areas are scanned at 10 μm intervals, the total test-line length is 100 mm for each area (100 electronic scans 1 mm in length). The stored intercept totals from each 1 mm^2 area were then transferred to a mainframe computer (Unix System) and mean AWUV values were calculated.

Values of Lm and AWUV were derived as follows:

Lm	=	Total test-line length/total number of intercepts.
V	=	volume (mm^3)
Surface area (mm^2)	=	2 V/Lm
AWUV (mm^2/mm^3)	=	2/Lm

2.2 Biochemical Procedures.

2.2.1 Tissue Preparation.

Lung samples were defatted by homogenisation in cold acetone followed by stirring overnight at 4°C. The homogenate was re-extracted once in acetone and dried under vacuum.

2.2.2 Determination of Total Collagen.

Total collagen was measured using 4-hydroxyproline (Hyp) as a relatively specific amino acid marker. Hyp accounts for approximately 13% dry weight of collagen (Bornstein and Traub, 1979). Minor amounts are also present in some other proteins, e.g. elastin (Sandberg *et al.,* 1981), C1q (a subcomponent of the first component of complement; Reid *et al.,* 1972), acetylcholinesterase (Rosenberry and Richardson, 1977), and pulmonary surfactant-associated glycoproteins (White *et al.,* 1985; Benson *et al.,* 1985; Ross *et al.,* 1986; Floros *et al.,* 1986). However, compared to collagen, the amounts are quantitatively insignificant. Three methods were used for Hyp determination, as described below.

2.2.2.1 Colorimetric Reaction for 4-Hydroxyproline.

Since Hyp is peptide bound, samples must be hydrolysed to release the Hyp for analysis. Samples were hydrolysed in, typically, 1.05 ml 6N hydrochloric acid (HCl) for 16 hr at 116°C in sealed tubes (12 mm x 100 mm). At the end of the reaction the HCl was removed by evaporation in a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, N.Y., USA) and the samples were reconstituted in 2 ml water.

The colorimetric reaction for Hyp is based on the oxidation of Hyp to pyrrole-2-carboxylic acid using chloramine T (Figure 2.3). Pyrrole-2-carboxylic acid is then reacted with p-dimethylaminobenzaldehyde (Ehrlich's reagent) to form a complex that is detected and quantified as a chromophore having an absorbance at 557nm. The colorimetric reaction does not detect 3-hydroxyproline due to its instability during oxidation (Szymanovicz *et al.*, 1978).

The method used was that of Woessner (1961), which is satisfactory for the determination of Hyp when applied to pure collagen or where the bulk of the non-collagenous proteins have been removed. The method cannot be used for samples containing less than 2% Hyp as the presence of other amino acids partially prevents the colour development.

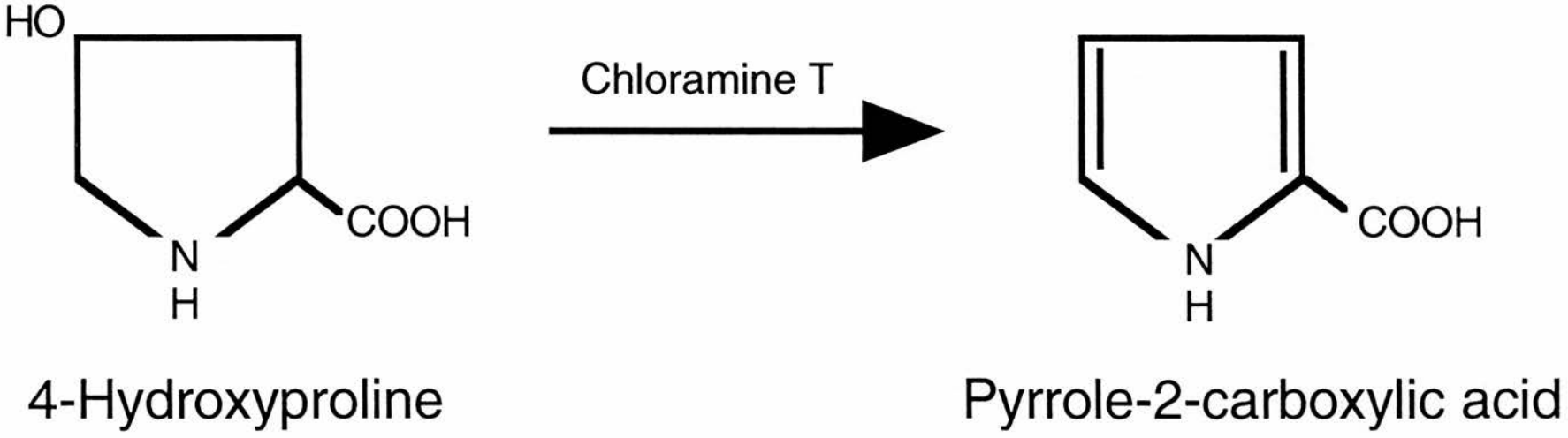


Figure 2.3 The oxidation of 4-hydroxyproline to pyrrole - 2 - carboxylic acid using chloramine T according to Peterkofsky and Prockop (1962).

The reagents were made up as follows:

Hyp standard. A stock solution of 10 µg/ml 4-Hydroxy-L-prolinewas prepared in distilled water and aliquots were frozen and kept at -30°C until required. Standards were prepared on the day of use by diluting the stock with water to obtain concentrations of 0.625 - 5 µg/2 ml. A reagent blank was also prepared containing 2 ml water.

Buffer. 50 g of citric acid monohydrate, 120 g sodium acetate trihydrate, and 34 g sodium hydroxide were dissolved in approximately 1 litre of distilled water. The pH was adjusted to 6.0 with glacial acetic acid and the final volume made up to 1 litre with distilled water. The buffer was stored at 4°C under toluene.

Chloramine T (Sodium p-toluenesulphonchloramide). A 0.05 M solution was prepared on the day of use by dissolving 0.705 g chloramine T in 10 ml water. 15 ml methyl cellosolve (2-methoxy ethanol) and 25 ml buffer were then added.

Perchloric acid. A 3.15 M solution was prepared by diluting 34 ml of 60% (v/v) perchloric acid to 100 ml with water.

p-Dimethylaminobenzaldehyde (Ehrlich's reagent). A 20% (w/v) solution was prepared on the day of use by dissolving 10g of p-dimethylaminobenzaldehyde in a final volume of 50 ml. Heating in a 60°C water bath aided solubilisation.

The procedure was as follows: Hyp oxidation was started by adding 1 ml chloramine T to each tube. The tubes were then mixed on a Vortex mixer and allowed to stand for 20 min at room temperature. The chloramine T was then destroyed by adding 1 ml perchloric acid, mixing and allowing to stand for 5 min. Finally, 1 ml p-dimethylaminobenzaldehyde solution was added and mixed until no Schlieren pattern was seen. The tubes were placed in a 60°C water bath for 20 min, then cooled in cold tap water for 5 min. The absorbance was determined at a wavelength of 557 nm. The amounts of Hyp in each sample were determined directly from a standard curve constructed with known concentrations of pure Hyp. After removal from the 60°C water bath and cooling, the colour is stable for at least 1 hr. The colour, however, decreases after 25 min of heating.

2.2.2.2 Determination of 4-Hydroxyproline on an Amino Acid Analyser.

As an alternative method for measuring Hyp in crude lung samples, an amino acid analyser was used. The method is based on the derivatisation of free amino groups, either primary or secondary, when exposed to phenylisothiocyanate (PITC) in the presence of a base, diisopropylethylamine (DIEA). PITC reacts quantitatively yielding a phenylthiocarbamyl-amino acid (PTC - AA). The PTC -AA derivatives have a strong UV absorbance at 254 nm.

The procedure was as follows: A known amount of nor-leucine (125nmoles) was added to the samples as internal standard prior to hydrolysis. The samples were dried in a Speed Vac Concentrator and placed in a vial (5 tubes/vial) containing 0.5 ml 6N HCl. The acid and vials were then purged with argon and the vials were sealed with a PTFE lined screw cap. Vapour phase hydrolysis was carried out at 110°C for 24 hr. The samples were reconstituted in 200µl 0.025% (w/v) K3ethylenediaminetetra-acetic acid (K3EDTA), filtered through a 0.22µm filter (Gelman Sciences Ltd., Northampton, England) and, after an appropriate dilution (1 : 100 with water), applied directly to a model 420 A amino acid analyser (Applied Biosystems Inc., Foster City, CA., USA) by Dr. A. D. Cronshaw (WelMet Protein Characterisation Facility, Department of Biochemistry, University of Edinburgh).

The amino acids were labelled automatically by the analyser and the resulting derivatives were separated on a 2.1 mm ID (internal diameter) x 22 cm cartridge-style reverse-phase column packed with C18 bonded phase silica. The peaks were resolved (Figure 2.4) by gradient elution as follows: 0-4 min, 0-2% buffer B; 4-10 min, 2-13% buffer B; 10-20 min, 13-35% buffer B; 20-25 min, 35-64% buffer B; 25-30 min, 64-100% buffer B. Buffer A was 50 mM sodium acetate, 3% (v/v) acetonitrile, final pH 5.22. Buffer B was 32 mM sodium acetate, 70% (v/v) acetonitrile, final pH 6.1. The eluate was monitored at 254 nm. The amounts of each amino acid in the samples were determined from their peak areas in relation to the peak areas produced by a standard containing known amounts (50 pmoles) of each amino acid plus 50 pmoles nor-leucine. The total amount of each amino acid in the original sample was then obtained after correcting for recovery of the internal standard of nor-leucine.

2.2.2.3 Determination of 4-Hydroxyproline by High Pressure Liquid Chromatography (HPLC) with Fluorimetric Detection.

As a final procedure for the determination of Hyp in crude lung samples, HPLC in conjunction with the specific derivatisation and detection of imino acids was employed. The method is based on the principle described by Einarsson (1985), in which primary amines are blocked by reaction with orthophthaldialdehyde (OPA) in the presence of β–mercaptoethanol, followed by labelling of the imino acids proline and Hyp via their secondary amine group with 9-fluorenylmethylchloroformate (FMOC-Cl).

The procedure was as follows: A known amount (160 µg) of 3,4-dehydroproline (3, 4-DHP) was added to each sample as internal standard. Samples were then hydrolysed in 1.05ml 6N HCl under nitrogen, in sealed tubes, for 24 hr at 110°C. The HCl was removed by evaporation and the samples were reconstituted in 200 µl 0.025% (w/v) K_3EDTA. The derivatisation procedure and chromatography conditions were essentially those of Teerlink *et al.* (1989). An appropriate amount of sample (5µl) was diluted in a total volume of 0.9 ml water. Then 0.15 ml of a 0.8 M sodium borate buffer (pH 9.5) was added, followed by 0.1 ml of acetonitrile containing 0.37M OPA and 0.37M β-mercaptoethanol. After 30s, 0.1 ml of a 0.75 M solution of iodoacetamide in acetonitrile was added to neutralise the excess of β-mercaptoethanol. After a further 30s, 0.3 ml of 5mM FMOC-Cl in acetone was added and immediately thereafter the reaction mixture was extracted twice with 2.0 ml of diethyl ether. The ether extracts were discarded. The lower phase was diluted with 1 ml of water and a 20 µl portion was chromatographed. Standard solutions containing known amounts of Hyp and a fixed amount of 3,4-DHP were treated in the same way as samples.

HPLC analysis was performed on a 4.6 mm ID x 25 cm Dynamax-300A column (Rainin Instrument Co. Inc., Woburn, MA., USA) packed with 5 µm C18 bonded spherical silica using a Gilson model 306 solvent delivery system and a Gilson model 231 autosampler (Gilson Medical Electronics S.A., Villiers-le-Bel, France). The peaks were resolved isocratically, at a flow rate of 1.0 ml/min, using 30% (v/v) acetonitrile in 50 mM acetic acid (adjusted, after mixing, to pH 4.3 with 1M NaOH). Fluorescence of the eluate was monitored with a Perkin-Elmer model LC-240 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) using an excitation wavelength of 260 nm and an emission wavelength of 330 nm. Results were calculated from calibration curves, constructed with known concentrations of pure Hyp versus the ratio of the peak area of Hyp to the peak area of 3, 4-DHP.

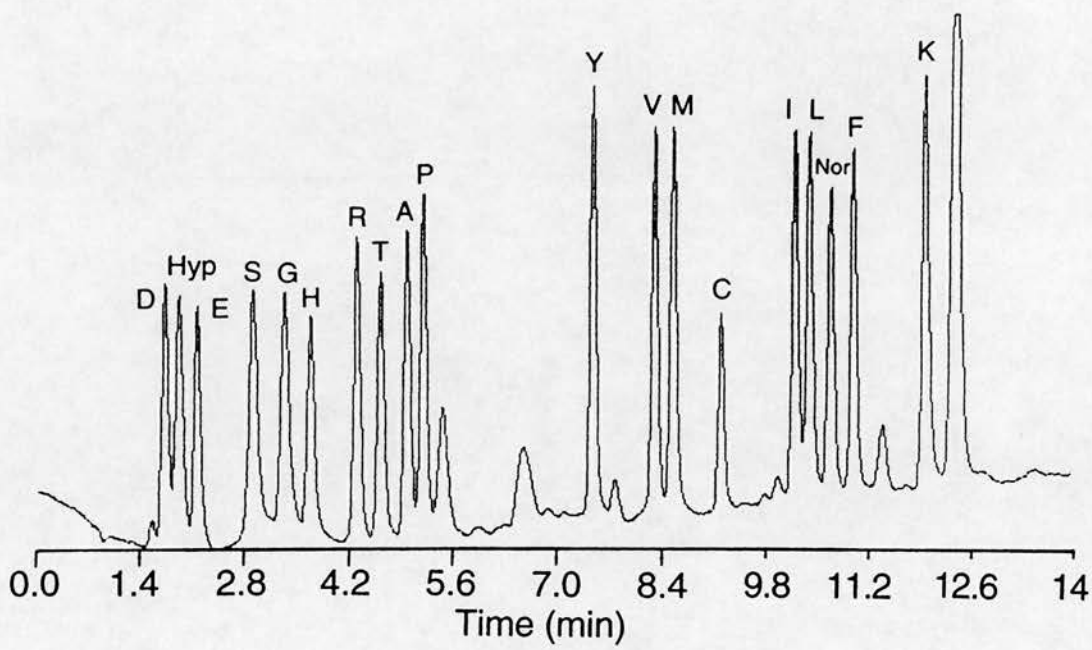


Figure 2.4 Separation of a standard mixture of amino acids on an Applied Biosystems model 420A amino acid analyser.

2.2.2.4 Calculation of Total Collagen Amounts from 4-Hydroxyproline.

Collagen standards for quantitation of the relative amounts of collagens I and III in lung (section 2.2.5.2) and for Enzyme Linked Immunosorbent Assay (ELISA; section 2.2.5.4), were quantified prior to use from their Hyp content as measured by the colorimetric assay. Hyp accounts for 14.2% of the dry weight of collagen I (Miller and Gay, 1982, 1987) and therefore a multiplication factor of 7.0 was used to convert the amount of Hyp to total collagen. Similarly, a multiplication factor of 5.8 was used for collagen III (17.25% Hyp) and a factor of 6.6 for collagen IV (15.12% Hyp; Miller and Gay, 1982, 1987).

2.2.3 Determination of Elastin.

Elastin was measured using the elastin-specific cross-links desmosine and isodesmosine which are separated, on the basis of differences in their hydrophobicity, on a C18 reverse-phase HPLC column followed by U.V. detection at 270nm.

In order to remove interfering substances the hydrolysate samples were treated by the cellulose "mini-column" method of Skinner (1982). The method is based on the selective adsorption of desmosine and isodesmosine onto cellulose in an organic acid-alcohol mixture followed by their elution with water. Mini columns were made from 3 ml plastic transfer pipettes (L.I.P. Equipment and Services Ltd., Shipley, England) by cutting off the top hemisphere of the bulb (which then becomes a 4 ml solvent reservoir) and plugging the tip with a single glass bead of 3.5 - 4.5 mm diameter. The barrel of the pipette was then clipped in a frame. A slurry was prepared by mixing 10g CF1 cellulose (Whatman Biosystems Ltd., Maidstone, England) with 200 ml of the mobile phase, n-butanol / acetic acid / water (4 : 1 : 1 v/v). The mixture was shaken to approximate homogeneity and then sonicated for 2-4 min to remove air bubbles. The columns were then packed to a height of 45 - 50 mm by pipetting 4 - 5 ml of the slurry into the bulb reservoir and allowing the cellulose to settle. A further 5 ml of mobile phase was added to wash fine particles from the bulb resevior. Samples, typically 120 - 150 µl of hydrolysate, were made up to a total volume of 0.5 ml with water and mixed, in order of addition, with acetic acid (0.5 ml), cellulose slurry (0.5 ml) and n-butanol (2 ml). The samples were transferred onto the prepared column and the tubes washed with 1.5 ml mobile phase which was also transferred to the columns. The columns were then washed with 15 ml mobile phase which removes interfering substances. Desmosine and isodesmosine were eluted from the column with water (5 ml). The samples were then

dried in a Speed Vac Concentrator, reconstituted in 100 µl water, filtered through a 0.22 µm filter and 10 µl was loaded onto the HPLC column.

The HPLC method used was essentially that of Yamaguchi *et al.* (1987) using the same column and solvent delivery system as for Hyp measurement (section 2.2.2.3). The peaks were resolved isocratically, at a flow rate of 0.8 ml/min, using 18% (v/v) acetonitrile / 82% (v/v) 0.1M sodium phosphate containing 20 mM sodium dodecyl sulphate (SDS), final pH 4.5. The eluate was monitored at 270nm with a model 759 U.V. absorbance detector (Applied Biosystems Inc., Foster City, CA., USA). Results were calculated from calibration curves, constructed with known concentrations of pure desmosine or isodesmosine (Elastin Products Co., Inc., Owensville, MO., USA) versus peak area.

2.2.4 Expression of Collagen and Elastin Data in Relation to Morphometry.

Biochemical measurements of Hyp and desmosine or isodesmosine were expressed per mm^3 of inflated tissue. These values were also divided by their corresponding adjacent slice AWUV values to give amounts per unit area of alveolar wall (i.e. per mm^2).

2.2.5 Analysis of Different Collagen Types.

2.2.5.1 Cleavage of Collagen by Cyanogen Bromide (CNBr).

Cynanogen bromide (CNBr) digestion in formic acid has been shown to solubilise the bulk of the total collagen from lung tissue (Laurent *et al.*, 1981a). Pepsin, on the other hand, solubilises less than 35% of total collagen in lung tissue (Seyer *et al.*, 1976; Reiser and Last, 1980), and this procedure may also selectively solubilise particular collagen types. CNBr was therefore used as the method of choice in extracting collagen from lung samples.

The action of CNBr upon proteins is unique in its selective attack on methionine (Gross, 1967). When methionine reacts with CNBr it is converted to homoserine lactone, which under acidic conditions is in equilibrium with homoserine (Figure 2.5). The formation of homoserine results in the liberation of the amino group of the residue that followed methionine. Accordingly, a residue of methionine which originally occupied an endo-position is converted to homoserine (lactone), which becomes the C-terminal residue of a peptide fragment. The reaction takes place under acidic conditions in order to denature the protein and to expose the side chains of methionine to attack by CNBr, which may be added in excess.

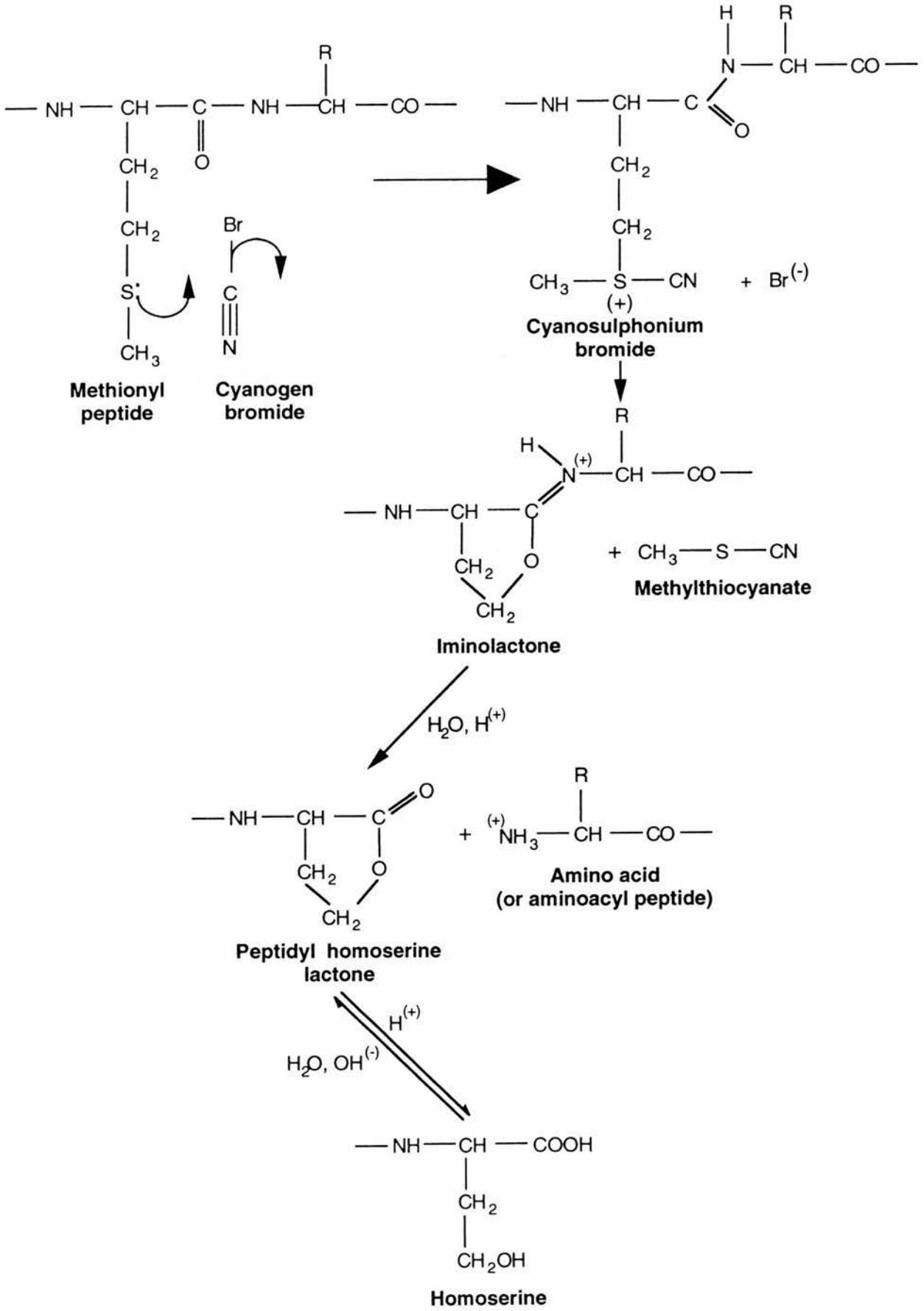


Figure 2.5 Cleavage of methionyl-X bonds with CNBr according to Gross (1967).

Excess reagent and by-products, such as methylthiocyanate, are volatile and readily removed by lyophilisation.

2.2.5.2 Determination of Collagens I and III in Lung.

The method of Laurent *et al.* (1981a) was applied to the quantitation of the relative amounts of collagens I and III in human lung. Approximately 50 mm^3 of agarose inflated lung parenchymal tissue was homogenised in a 1 ml all glass homogeniser (Fisons Instruments, East Grinstead, England) with 1 ml of 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) at 4°C, and then transferred to a 10 ml Teflon centrifuge tube (Nalgene, supplied by Merck Ltd., Glasgow, Scotland). The homogeniser was then washed with a further 4 ml of PBS which was also transferred to the centrifuge tube. The homogenate was centrifuged at 4000 g for 5 min and the residue was rehomogenised in 2% sodium dodecyl sulphate (SDS) at room temperature. The homogenate was again centrifuged and the SDS extraction repeated a further four times. The remaining residue was then extracted three times with PBS to remove the unbound SDS. The residue was then rehomogenised in acetone, centrifuged at 4000 g for 5 min and the supernatant discarded. This step was repeated and the pellet was dried under vacuum.

The dried pellet was homogenised in a 1 ml all glass homogeniser in 0.6 ml of 70% formic acid and the homogenate was transferred to a 10 ml Teflon centrifuge tube. The homogeniser was washed with a further 0.6 ml of 70% formic acid which was also transferred to the centrifuge tube. A further 0.3 ml of a 50 mg/ml solution of CNBr in 70% formic acid was then added. Nitrogen gas was bubbled through the homogenate and the tubes were sealed. The tubes were mixed end-over-end for 6 hr in a 25°C incubator (Lab-Therm, Adolf Kühner AG., Basel, Switzerland). At the end of the reaction, the digest was centrifuged at 5000 g for 20 min to pellet insoluble material. The supernatant was collected, diluted 15-fold with water and lyophilised.

Collagen I from rat tail and collagen III from rat skin, both purified and kindly donated by Dr. D.R. Shackleton and Dr. J.R.E. McBeath respectively (Department of Biochemistry, University of Edinburgh), were used as standards. Known amounts, previously quantified using the colorimetric assay of Woessner (1961) to measure Hyp (section 2.2.2.1), were digested using a three-fold excess (w/w) of CNBr in 70% formic acid. Nitrogen gas was bubbled through the mixture and the tubes were sealed. The tubes were then mixed end-over-end for 6 hr in a 25°C incubator. The digest was diluted 15-fold with water and lyophilised.

CNBr peptides in both tissue extracts and collagen standards were analysed by discontinuous SDS-PAGE using a separating gel of 12% acrylamide (section 2.2.5.3). Equal amounts of the collagen standards were mixed and dilutions run singly. Samples were run in duplicate.

2.2.5.3 Discontinuous SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE).

The relative amounts of the major lung collagens (I and III) were determined using discontinuous SDS-PAGE to separate the peptides generated by CNBr digestion. Discontinuous SDS-PAGE is a widely used technique which separates proteins according to their molecular weight. SDS is a negatively charged detergent that binds to proteins such that approximately 0.5 mol of SDS is bound per amino acid residue. This large amount of negatively charged detergent molecules is sufficient to overwhelm the intrinsic charge on the polypeptide chain so that the net charge per unit mass becomes approximately constant. In the presence of SDS and the reducing agent β-mercaptoethanol (which reduces disulphide bonds) proteins are denatured and adopt an extended coil configuration. Electrophoretic migration is then determined by the effective molecular length or approximate molecular weight of the polypeptide chain. Molecular weights can be estimated from a plot of distance migrated versus log molecular weight, using proteins of known molecular weight as standards.

Collagenous proteins behave anomalously in SDS-PAGE in comparison to typical globular proteins (Furthmayr and Timpl, 1971). The collagen I α chains migrate with apparent molecular weights of 133,000 for α1 and 122,000 for α2 compared with the true values of 96,000 for each chain. The different mobilities of the α1 and α2 chains in SDS-PAGE could be due to localised conformation effects resulting from restricted rotation of polypeptide chains about imino acid residues (Furthmayr and Timpl, 1971).

Discontinuous SDS-PAGE was performed in vertical slab gels using the buffer system of Laemmli (1970). Gels containing 4.5% (stacking gel), 6.0% or 12% (separating gel) acrylamide were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N, N'-bis-methylene acrylamide. The final concentrations in the separating gel were as follows: 0.375M Tris-HCl (pH 8.8) and 0.2% (w/v) SDS. The gels were polymerised chemically by the addition of tetramethylethylenediamine (TEMED; 25µl/50ml separating gel) and 2% ammonium persulphate (1.25ml/50ml separating gel). Gels 13 cm x 14 cm were prepared in 16 cm x 18 cm glass plates with 1.5 mm spacers. The stacking gels of height 2 cm

contained 0.125M Tris- HCl (pH 6.8) and 0.2% SDS (w/v) and were polymerised chemically by the addition of TEMED (25µl/25ml stacking gel) and 2% ammonium persulphate (0.625ml/25ml stacking gel). The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS (w/v). The samples contained the final concentrations : 0.1M Tris-HCl (pH 6.8), 2% (w/v) SDS , 10% (v/v) glycerol, 3% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue as the dye. The proteins were completely denatured by immersing the samples, in microcentrifuge tubes, for 3.0 min in boiling water. The samples were then loaded into the wells using a 100 µl microsyringe (Hamilton Bonaduz AG., Bonaduz, Switzerland). Electrophoresis was carried out at 40mA constant current per gel until the bromophenol blue marker reached the bottom of the gel (about 3 1/2 hr). Gels were fixed and stained simultaneously overnight in 250 ml 8% (v/v) acetic acid/50% (v/v) methanol containing 0.1% (w/v) Coomassie Brilliant Blue R-250. Gels were then destained over 5-6 hr by continuous shaking in several changes of 8% (v/v) acetic acid.

Densitometric scanning of the gels was performed at 530 nm using a Chromoscan 3 scanning densitometer (Joyce-Loebl, Gateshead, England), linked to an IBM-compatible microcomputer, and having a slit width of 0.3 mm x 5 mm. Peaks were integrated using the Chromoscan 3 External Data Analysis Package.

2.2.5.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Collagen IV.

As collagen IV is a relatively minor component of the lung, the sensitivity afforded by an immunological technique was considered to be the most suitable method for its quantitation. An attempt was therefore made to apply an ELISA to the quantitation of the peptides of collagen IV resulting from CNBr digestion. The technique used was based on those of Rennard *et al.* (1980b) and Bellon (1985). In principle, purified antigen is first allowed to adsorb to flat-bottomed microtitre wells. Next, an antibody/soluble antigen mixture is incubated with the insolubilised antigen. In a non-equilibrium assay, antibody and soluble antigen are pre-incubated together, usually overnight, before their addition to the microtitre wells containing the insolubilised antigen. In an equilibrium assay, antibody and soluble antigen are added to the microtitre wells containing the insolubilised antigen without pre-incubation. The wells are washed and a second antibody (raised against the first antibody and covalently linked to an enzyme) is added, allowed to bind and then the unbound excess second antibody is removed. Finally, a substrate for the bound enzyme is added to

the wells and the subsequent reaction produces a chromophore. Incubation of first antibody with soluble antigen inhibits binding to the insolubilised antigen. The amount of inhibition depends on the amount of soluble antigen present. Therefore, incubation of antibody with known amounts of soluble antigen allows the construction of a standard curve from which amounts of antigen in samples can be determined.

The procedure used for the ELISA was as follows: CNBr peptides of purified human collagen IV (Sigma Chemical Co., Poole, England) were dissolved in 20 mM sodium carbonate buffer, pH 9.6, and 100 µl was added to each well of a microtitre plate (NUNC., Gibco Ltd., Paisley, Scotland). Adsorption was allowed to take place overnight in a humidified atmosphere at 4°C. The coated plate was then washed three times with 20 mM PBS, pH 7.4, containing 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20. This buffer was also used to dilute anti-collagen IV antibody and the soluble collagen IV peptides. In the equilibrium assay, 50 µl of soluble peptides were added to the antigen-coated wells followed by the addition of 50 µl of antibody. The mixture was then incubated at room temperature for 2 hr. In the non-equilibrium assay, antibody and soluble peptides were incubated together overnight at 4°C. A 100 µl aliquot of the pre-incubation mixture was then added to the antigen-coated wells followed by incubation at room temperature for 2 hr. The plates were then washed as before. The second antibody (covalently linked to horseradish peroxidase, HRP) was diluted in the above buffer and 100ml was added to each well followed by incubation at room temperature for 2 hr. The plate was washed and 200 µl of substrate for HRP was then added to each well and the reaction allowed to proceed for 1 hr in the dark at room temperature. The substrate used for HRP was O-phenylenediamine dissolved in methanol (10 mg/ml) and diluted 1 : 100 into 0.03% (v/v) hydrogen peroxide (H_2O_2) in distilled water. The substrate was prepared fresh on the day of use. The reaction was stopped by the addition of 50 µl of 4N sulphuric acid. The absorbance at 490nm was measured directly through the microtitre wells using a microtitre plate reader (Dynatech Laboratories Ltd., Billinghurst, England).

A number of antibodies were compared, all of which had been raised against native human collagen IV. Two polyclonal antibodies were raised in rabbits, one was a gift from Dr. V. Duance (AFRC, Bristol), the other was obtained commercially (Euro-diagnostics BV., Apeldoorn, Holland). One affinity purified antibody was raised in goats (Southern Biotechnology Associates, Inc., Birmingham, AL., USA). Two mouse monoclonal antibodies were also tested, both obtained commercially (Serotec, Oxford, England and Euro-diagnostics BV., Apeldoorn, Holland).

HRP-coupled anti-species antibodies were obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke. Anti-rabbit antibody and anti-goat antibody had been raised in donkey, and anti-mouse antibody had been raised in sheep.

Anti-collagen IV antibodies (Euro-diagnostics BV.) were also purified by immunoaffinity chromatography against CNBr peptides of human collagens I and III (Sigma Chemical Co., Poole, England). The method used was a modification of the method described by Wick *et al.* (1979). Lyophilised CNBr peptides of collagens I and III (500µg of each type), were dissolved in 1 ml 0.1M sodium phosphate buffer, pH 8.0, containing 0.4M NaCl. A further 2 ml of a pre-washed suspension of CNBr activated Sepharose 4B (Pharmacia Biosystems Ltd., Milton Keynes, England), in the above buffer, was added. The reaction was then allowed to proceed for 2 days at 4°C. The Sepharose was kept in suspension by continuously rotating the tube end-over-end. At the end of the incubation period, the reaction mixture was transferred to a Poly-Prep column (Bio-Rad Laboratories Ltd., Hemel Hempstead, England), and the reaction stopped by filtration of the gel and a subsequent incubation with 0.1M Tris-base for 2 hr at room temperature. The adsorbents were then washed successively, at 4°C, with 10 ml each of 0.1M sodium phosphate, pH 8.0, PBS, pH 7.2, 1 M acetic acid, 0.05M HCl containing 0.15 M NaCl and finally again with PBS. 100 µl of antiserum (Euro-diagnostics BV.), was added to the top of the column, allowed to run into the gel and then washed in with approximately 900 µl PBS. After 1 hr the unbound material was collected with a PBS wash. The antibodies were then concentrated to approximately their original volume in an Amicon Micro-concentrator (Amicon Ltd., Stonehouse, England).

2.2.6 Statistical Analysis.

Statistical analyses were performed using the Apple Macintosh versions of the programs Cricket Graph and Minitab. The product-moment correlation coefficient (r) was considered significant when $p < 0.01$. The Analysis of Variance ratio (F) and the Mann-Whitney rank sum test were considered significant when $p < 0.05$.

CHAPTER 3. DEVELOPMENT OF TECHNIQUES

3.1 Elastin.

Two previous studies of the elastin content in various tissues used reverse-phase High Performance Liquid Chromatography (HPLC) to measure desmosine and isodesmosine (Yamaguchi *et al.*, 1987; Schwartz *et al.*, 1990). In both studies, elution was achieved isocratically using a phosphate buffer/acetonitrile mixture containing 20 mM SDS, final pH 4.5. However, because the reverse-phase columns used in these studies differed in both manufacturer and dimensions, the acetonitrile concentration needed to achieve separation of desmosine and isodesmosine in a reasonable time differed markedly. Yamaguchi *et al.* (1987) used 36% acetonitrile, and Schwartz *et al.* (1990) used 10 % acetonitrile. Therefore, in order to determine the concentration of acetonitrile needed to achieve separation of desmosine and isodesmosine in a reasonable time, a linear gradient from 0% acetonitrile to 30% acetonitrile was used initially. Buffer A consisted of 0.1M sodium phosphate containing 20mM SDS, final pH 4.5. Buffer B consisted of 30% acetonitrile / 70% 0.1M sodium phosphate containing 20 mM SDS, final pH 4.5. Gradient elution from 0% buffer B to 100% buffer B was achieved at a flow rate of 0.8 ml/min over 30 mins (Figure 3.1). From Figure 3.1, the concentration of acetonitrile needed to achieve optimum separation of desmosine and isodesomine was estimated to be 18%, i.e. the concentration of acetonitrile mid-way between the two peaks. This was confirmed by repeating the separation isocratically using 18% acetonitrile (Figure 3.2).

The chromatogram produced with a crude tissue hydrolysate was complex (Figure 3.3). Identification of desmosine and isodesmosine peaks was difficult and quantitation was not possible due to their poor resolution from contaminating peaks. Like Yamaguchi *et al.* (1987), the cellulose "mini-column" method of Skinner (1982) was used therefore to purify desmosine and isodesomine from the crude hydrolysate prior to chromatography. Figure 3.4 shows a typical chromatogram produced from a "purified" hydrolysate, i.e. after pre-treatment on the cellulose mini-column. Skinner (1982) demonstrated recoveries of between 99% and 102% following purification of isodesmosine hydrolysed in the presence of either gelatin or bovine serum albumin.

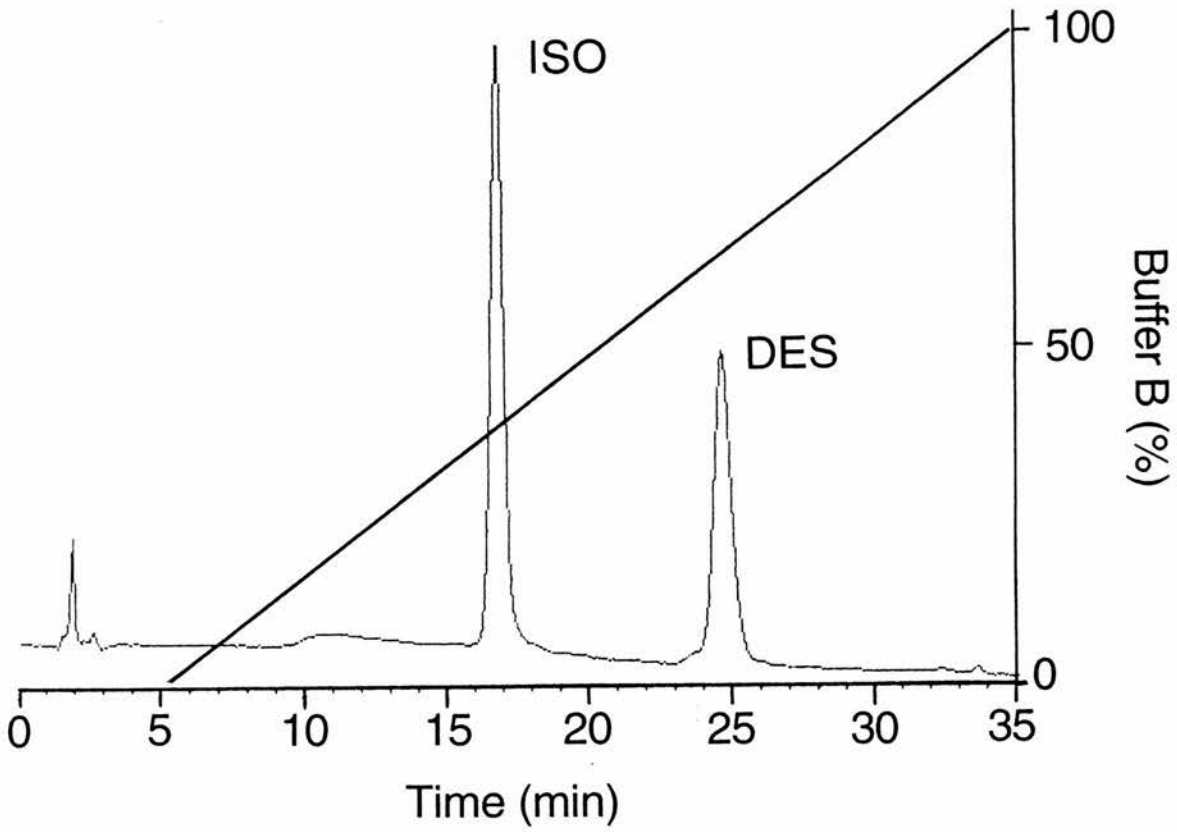


Figure 3.1 Separation of isodesmosine (ISO) and desmosine (DES) on a reverse-phase C18 HPLC column by gradient elution from 0% buffer B to 100% buffer B. Buffer A consisted of 0.1M sodium phosphate containing 20mM SDS, final pH 4.5. Buffer B consisted of 30% acetonitrile / 70% 0.1M sodium phosphate containing 20mM SDS, final pH 4.5.

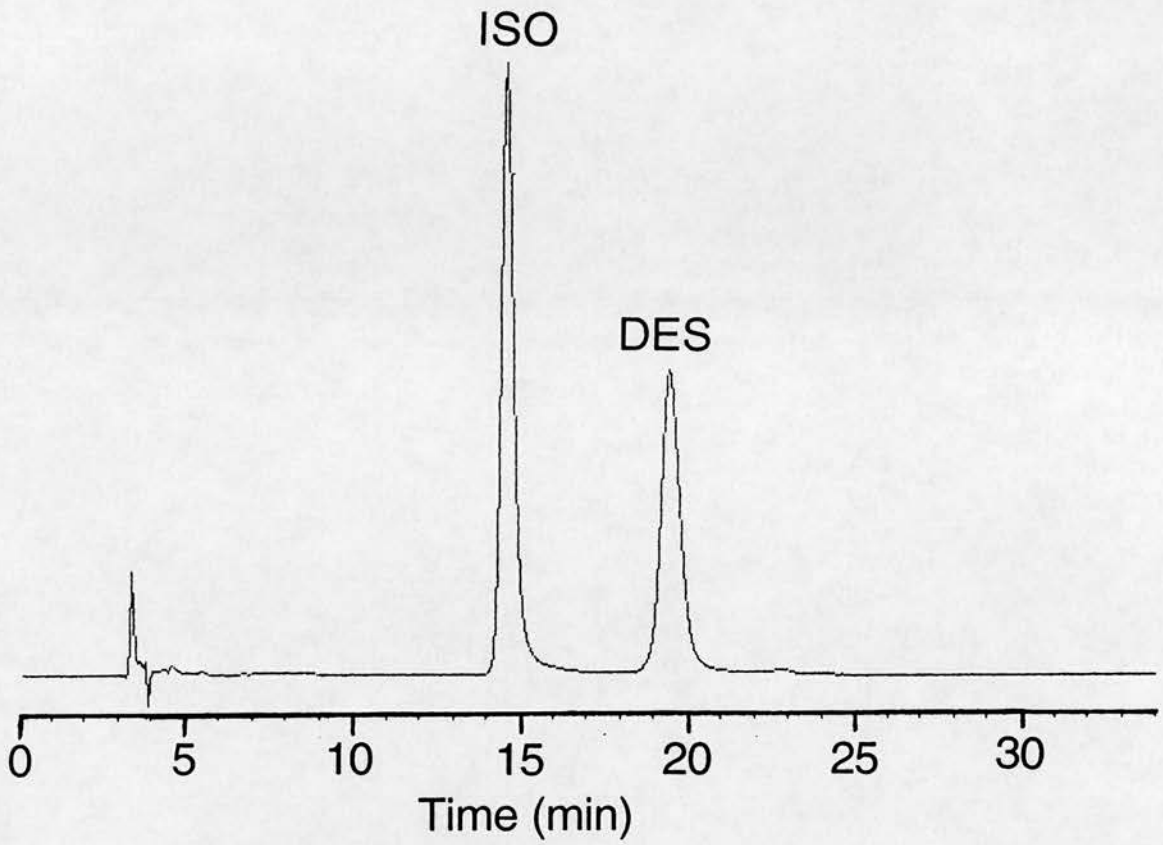


Figure 3.2 Separation of isodesmosine (ISO) and desmosine (DES) on a reverse-phase C18 HPLC column by isocratic elution with 18% acetonitrile / 82% 0.1M sodium phosphate containing 20mM SDS, final pH 4.5.

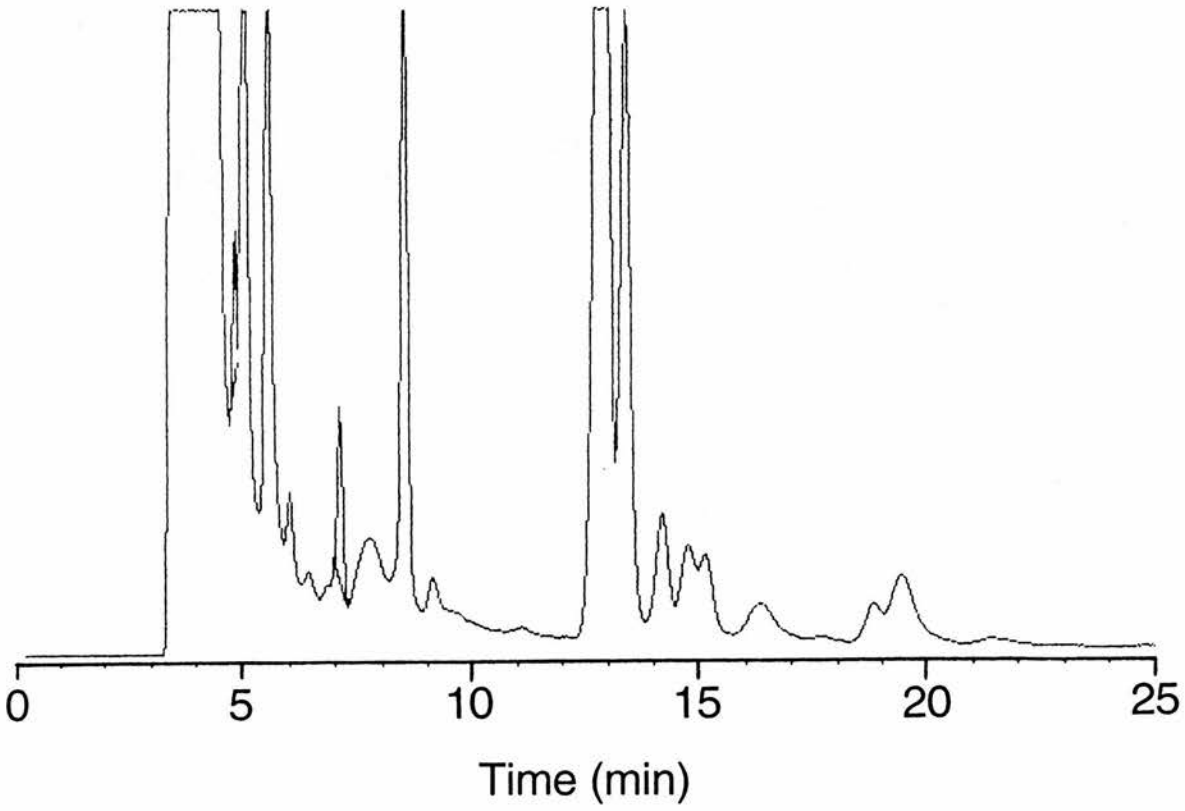


Figure 3.3 Chromatogram of a crude lung hydrolysate sample injected onto a reverse-phase C18 HPLC column and eluted isocratically with 18% acetonitrile / 82% 0.1M sodium phosphate containing 20mM SDS, final pH 4.5.

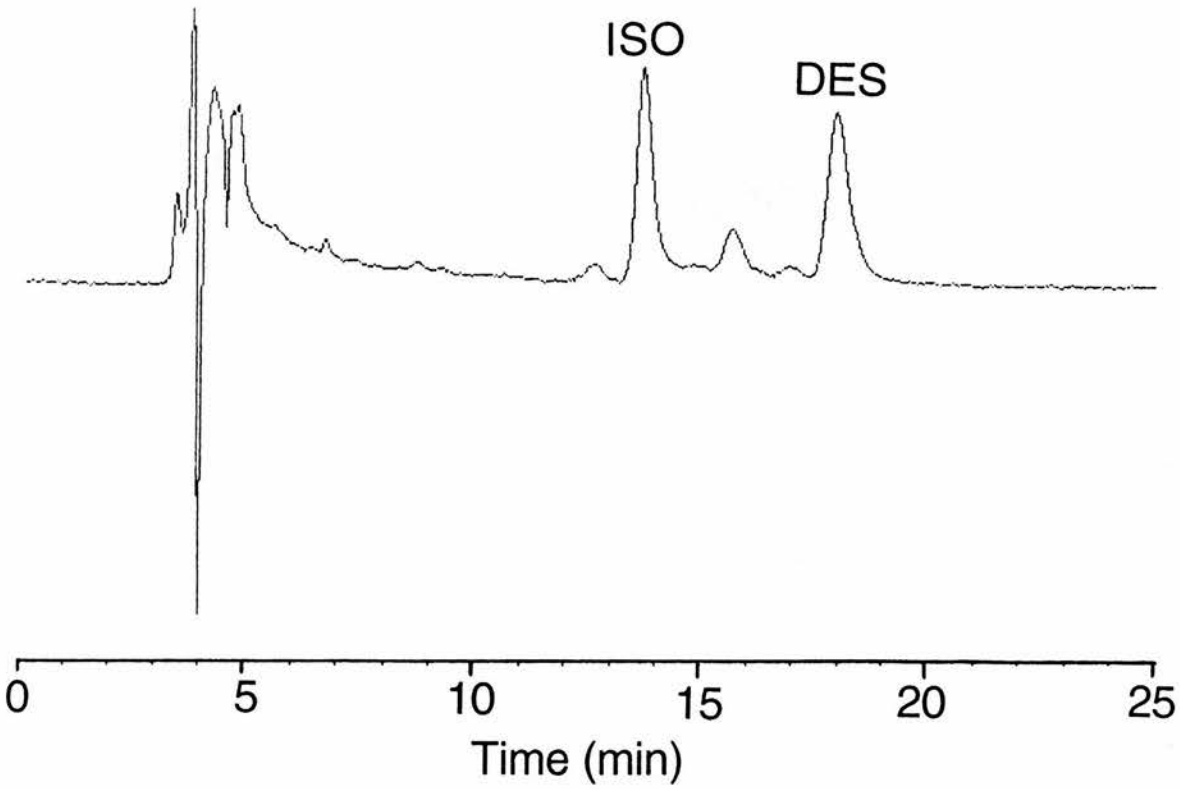


Figure 3.4 Chromatogram of a lung hydrolysate sample after purification on a cellulose mini-column (section 2.2.3) and injection onto a reverse-phase C18 HPLC column followed by isocratic elution with 18% acetonitrile / 82% 0.1M sodium phosphate containing 20mM SDS, final pH 4.5. ISO = isodesmosine, DES = desmosine.

3.2 Collagen.

The method for Hyp determination by reverse-phase HPLC with fluorimetric detection (section 2.2.2.3) was essentially that of Teerlink *et al.* (1989). However, a modification to the published method was found to be necessary. It was shown that isocratic elution using 34% acetonitrile failed to resolve the proline peak from the FMOC-OH peak completely (Figure 3.5), whereas 30% acetonitrile resulted in a better resolution of these peaks. Figure 3.6 shows the separation of the FMOC derivatives of Hyp from 3, 4-DHP, with retention times of 8.22 min and 20.64 min respectively. At 27.27 min, FMOC-OH, the hydrolysis product of FMOC-Cl, is well resolved from proline, which has a retention time of 33.48 min.

3.3 Quantitation of the Relative Amounts of Collagens I and III.

Preliminary data on the relative amounts of collagens I and III in lung tissue were obtained using the method of Laurent *et al.* (1981a). These procedures have been reported to remove the bulk of potentially contaminating non-collagen proteins (Laurent *et al.*, 1981a). A typical separation of CNBr peptides by discontinuous SDS-PAGE is shown in Figure 3.7.

Quantitation of the relative amounts of collagens I and III in lung tissue was accomplished by determining the relationship between known amounts of CNBr fragmented collagen standards applied to the gels and the relative sizes of peaks corresponding to CNBr peptides distinctive for each collagen type. Such a relationship is shown for two bands identified, with reference to the literature (Laurent *et al.*, 1981a; Kelley *et al.*, 1984; Kirk *et al.*, 1984a; Bateman *et al.*, 1986), as α1(I)-CB-8 and α1(III)-CB-5 (Figure 3.8). The plots were linear with correlation coefficients of 0.984 or greater. The effect of a fixed amount of one collagen type on the standard curve of the other collagen type was not evaluated. However, from Figures 3.7 and 3.12, it seems unlikely that the standard curve of one collagen type in the presence of a fixed amount of the other collagen type would be significantly displaced. The relative amounts of collagens I and III in the lung extracts were then determined from the peak sizes of these two bands in relation to the standard curve. Using the peptides α1(I)-CB-8 and α1(III)-CB-5, the relative amounts of collagen types I and III were quantified in eight lungs. Results were obtained from four separate gels, each with its own set of standards.

The mean value for the amount of collagen III as a proportion of the total amount of collagens I + III was found to be 0.77 ± 0.1 (SEM; n = 8). This value is at variance with previous results from human lung. For example, Seyer *et al.* (1976) and Kirk *et al.* (1984a) also analysed CNBr extracts of lung and found the proportion of collagen III to be 0.33 and 0.29, respectively. A possible explanation for the present findings is incomplete digestion of the collagen III standard by CNBr. It can be seen from the gel (Figure 3.7) that there are a number of higher molecular weight components present in the collagen standards which are not present in the lung extracts. These components may include higher molecular weight peptides in which α1(III)-CB-5 is still incorporated. As the standards and samples were reduced prior to electrophoresis the α1(III)-CB-5 band may be contaminated with α1(III)-CB-9 which under non-reducing conditions runs with higher molecular weight components as it is known to be involved in intra-molecular disulphide bridges (G.L. Laurent, personal communication).

In the course of this study it was found that collagen could not be extracted by CNBr from lung tissue which had been stored in 70% ethanol for long periods of time (approximately two years). With fresh agarose inflated lungs (section 4.3) it was found that the mean total Hyp content of 50 mm^3 samples was 227 nmol (SD 158 nmol; n = 102). CNBr soluble fractions from equivalent samples (n = 25) stored in 70% ethanol for approximately two years were found to contain less than 4.77 nmol Hyp by the method of Woessner (1961; section 2.2.2.1). This represents less than 2.1% of extractable Hyp. The samples used in this preliminary study had been stored in 70% ethanol for approximately 12 weeks. One explanation for reduced extraction efficiency is failure of cleavage of collagen molecules due to atmospheric oxidation of methionine residues during storage. It is known that methionine sulphoxide and methionine sulphone are not converted to homoserine by CNBr (Gross, 1967). Methionine sulphoxide and methionine sulphone can be quantitated after alkaline hydrolysis followed by amino acid analysis (Ray and Koshland, 1962). This represents, therefore, a means whereby the effects of storage on the conversion of methionine to methionine sulphoxide/sulphone could be investigated.

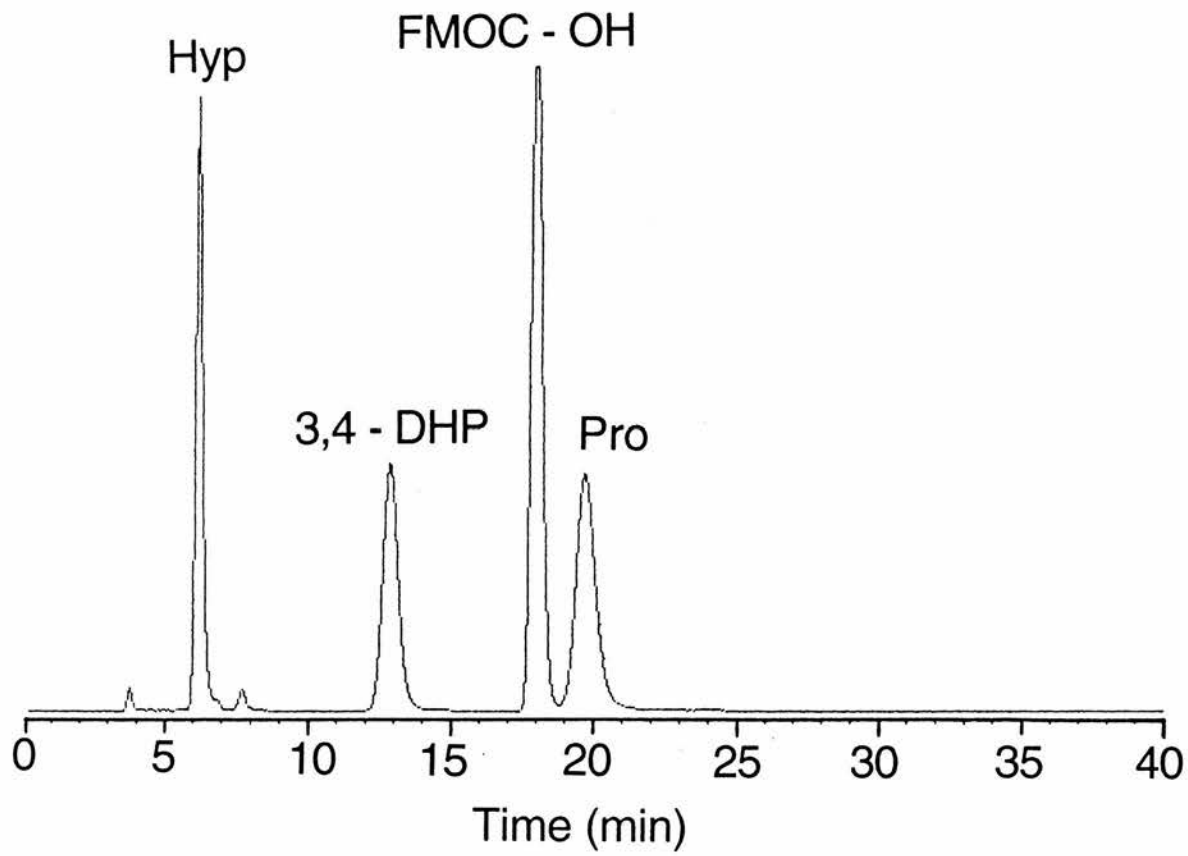


Figure 3.5. Separation of Hyp, 3, 4-DHP, FMOC - OH and proline (Pro) on a reverse-phase C18 HPLC column by isocratic elution with 34% acetonitrile in 50mM acetic acid, final pH 4.3.

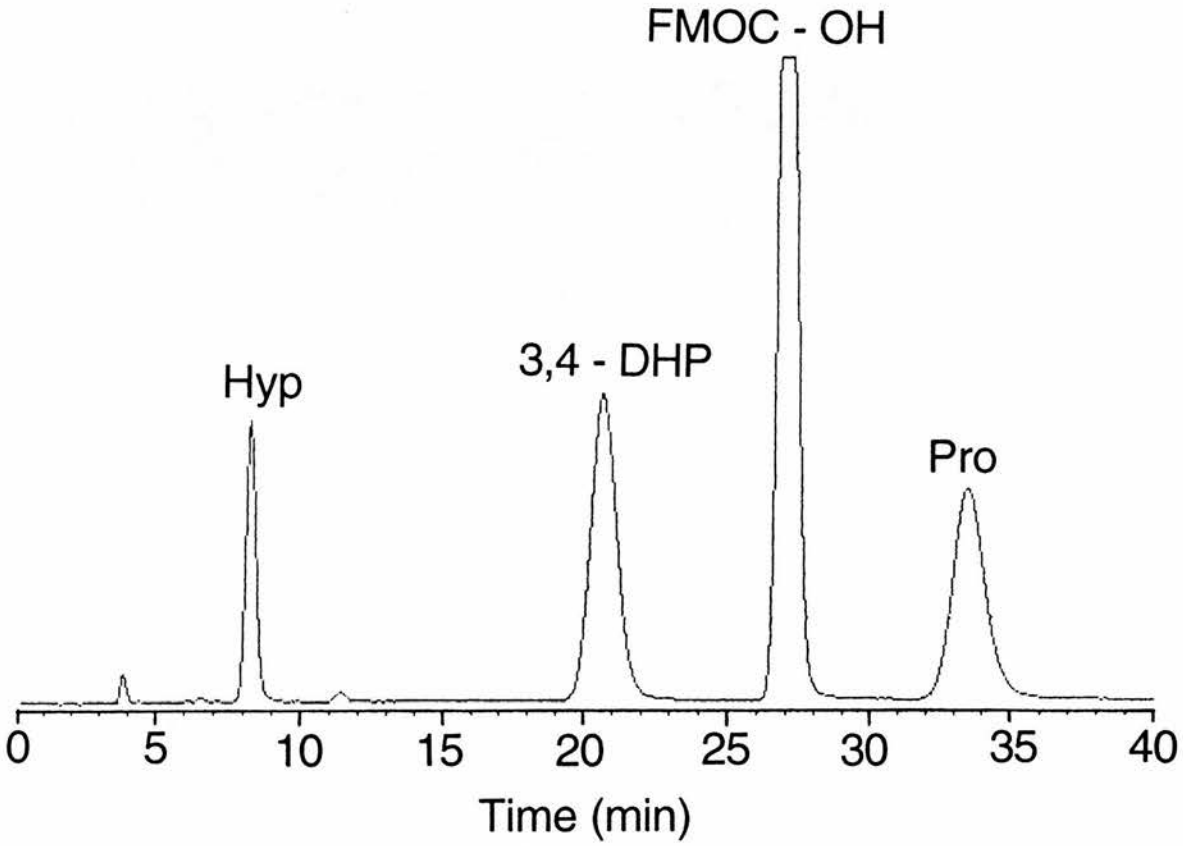


Figure 3.6 Separation of Hyp, 3,4 - DHP, FMOC-OH and proline (Pro) on a reverse-phase C18 HPLC column by isocratic elution with 30% acetonitrile in 50mM acetic acid, final pH 4.3.

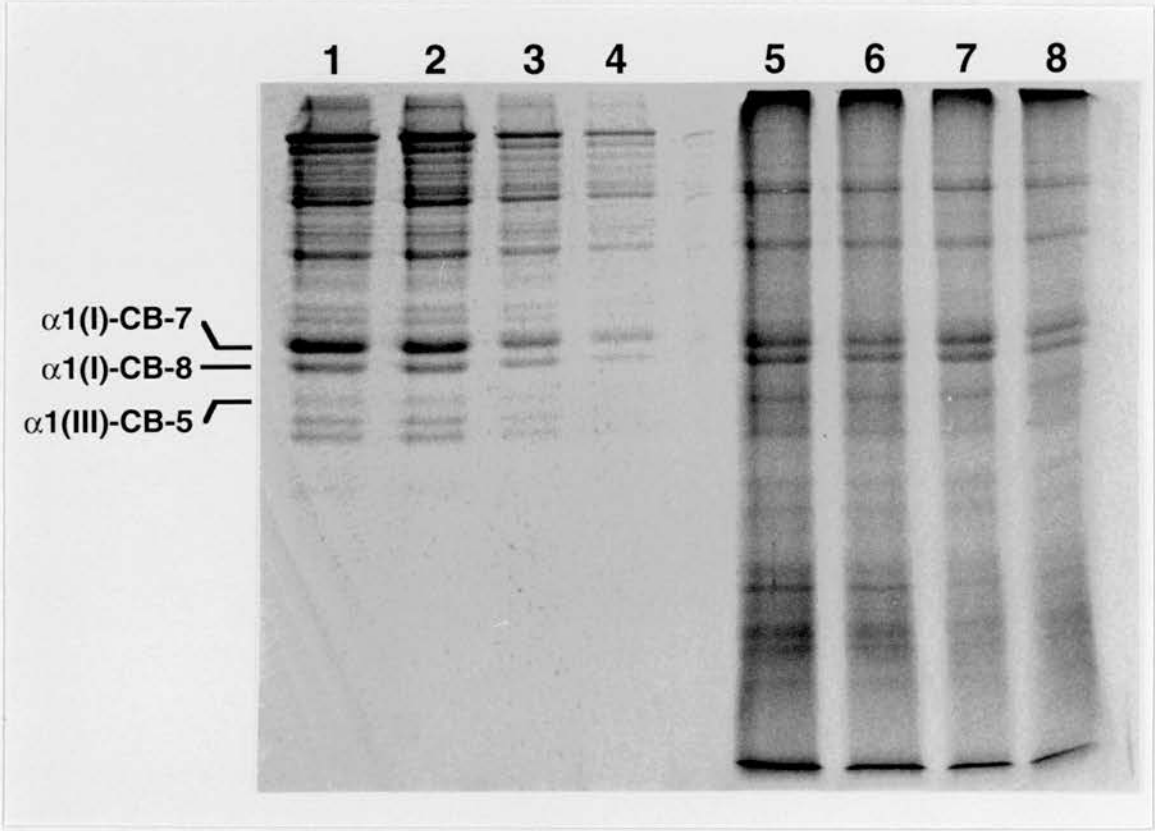


Figure 3.7 Discontinuous SDS-PAGE of CNBr digests of collagen I and collagen III standards and extracts of agarose inflated lung tissue. Tracks 1-4, mixed collagen I and collagen III standards from rat tail and rat skin respectively; Track 1, 50 μg collagen I + 50 μg collagen III; Track 2, 40 μg collagen I + 40 μg collagen III; Track 3, 20 μg collagen I + 20 μg collagen III; Track 4, 10 μg collagen I +10 μg collagen III. Tracks 5 - 8, extracts of agarose inflated lung tissue. Tissue samples were treated prior to CNBr digestion using the SDS extraction procedure of Laurent *et al.* (1981a). Standards and tissue samples were reduced prior to electrophoresis.

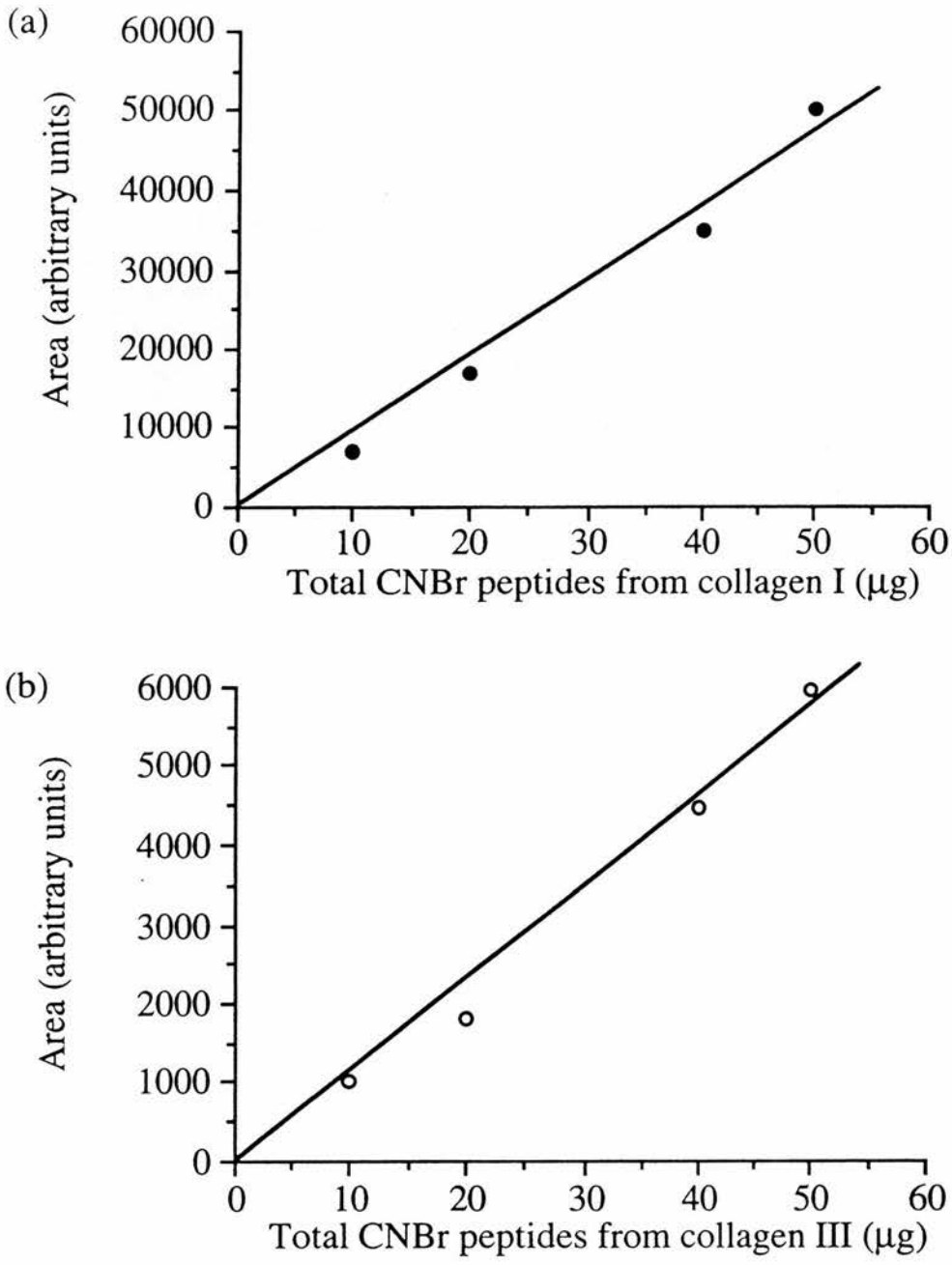


Figure 3.8 Relationship between peak areas for individual CNBr peptides and total amounts of CNBr digested collagen standards analysed by SDS-PAGE. (a) α1 (I) - CB - 8; (b) α1 (III) - CB - 5.

3.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Collagen IV.

3.4.1 Antibody/Antigen Optimisation.

In the ELISA for collagen IV the amount of peptides used for coating the well and the concentration of primary antibody were determined simultaneously in order to optimise assay conditions. For this purpose wells were coated with serial dilutions of CNBr peptides from collagen IV and subsequently incubated for 2 hr with serial dilutions of antiserum. The amounts of peptide and antiserum were chosen to generate 0.8 - 1.0 absorbance unit. A typical optimisation assay for collagen IV, using the antiserum supplied by V. Duance (AFRC, Bristol), is shown in Figure 3.9. For this particular assay, a peptide amount of 7.3 μg per well and an antiserum dilution of 1:500 were chosen which gave an absorbance of 1.0.

The effect of equilibrium on the assay was examined in order to improve the sensitivity. In the equilibrium assay, soluble antigen and antiserum were added simultaneously to the antigen-coated wells which allowed soluble and insoluble antigen to compete directly for binding to the primary antiserum. In the non-equilibrium assay, soluble antigen and antiserum were incubated together at 4°C overnight before being added to the antigen-coated wells. The 50% inhibitory level was calculated from standard curves plotted using the software package Assayzap (Dr. P. Taylor, MRC, Centre for Reproductive Biology, Edinburgh). Under non-equilibrium conditions, the sensitivity of an assay using the antiserum supplied by V. Duance was greater, as illustrated in Figure 3.10. This increase in sensitivity is reflected in a shift of the standard curve to the left. The 50% inhibitory level for the non-equilibrium assay was 0.19 μg/100 μl and for the equilibrium assay approximately 0.70 μg/100 μl. There appeared to be no depression of binding between 0.4 μg/100μl and 0.8 μg/100 μl with the equilibrium assay which would again reflect a lack of sensitivity. All subsequent assays were performed under non-equilibrium conditions.

3.4.2 Cross-reactivities.

The cross-reaction of CNBr peptides of collagen I and collagen III with a number of anti-collagen IV antisera was assessed from standard curves produced by reacting anti-collagen IV antisera with serial dilutions of CNBr peptides from purified collagens I, III and IV in a non-equilibrium ELISA. The standard curves produced by a typical cross-reactivity experiment are shown in Figure 3.11. Cross-reaction was quantified at the 50% inhibitory level (Abraham, 1969) and the results are summarised in Table 3.1. All antisera tested cross-reacted to varying degrees with the CNBr peptides of collagen I and collagen III.

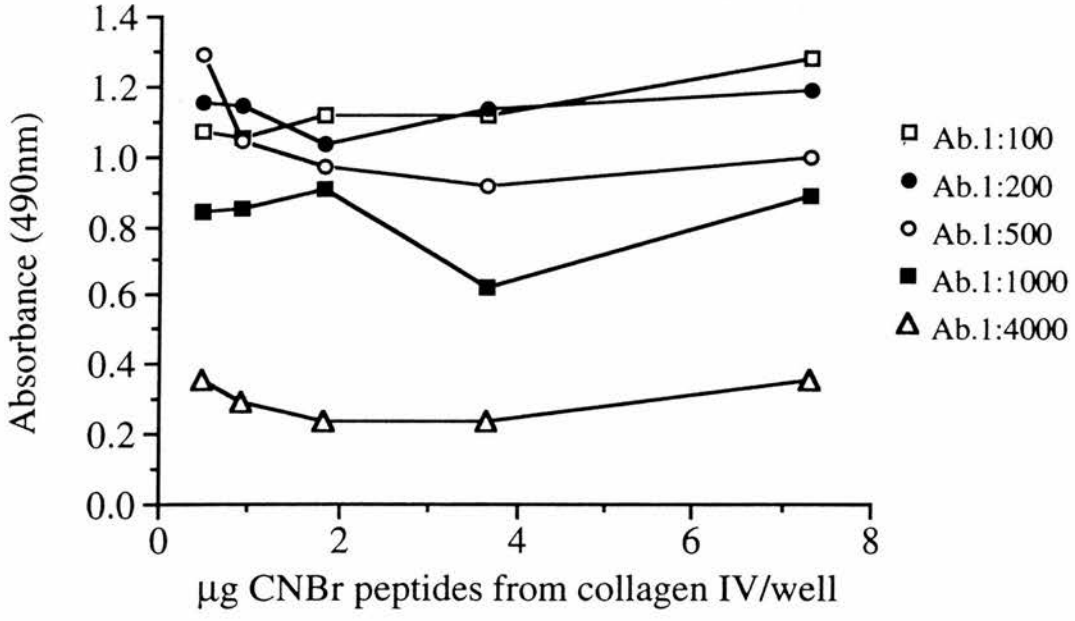


Figure 3.9 Optimisation of antibody (Ab) dilution and amount of CNBr peptides coating each microtitre plate well in an ELISA for collagen IV. Each point on the graph represents the mean of two determinations.

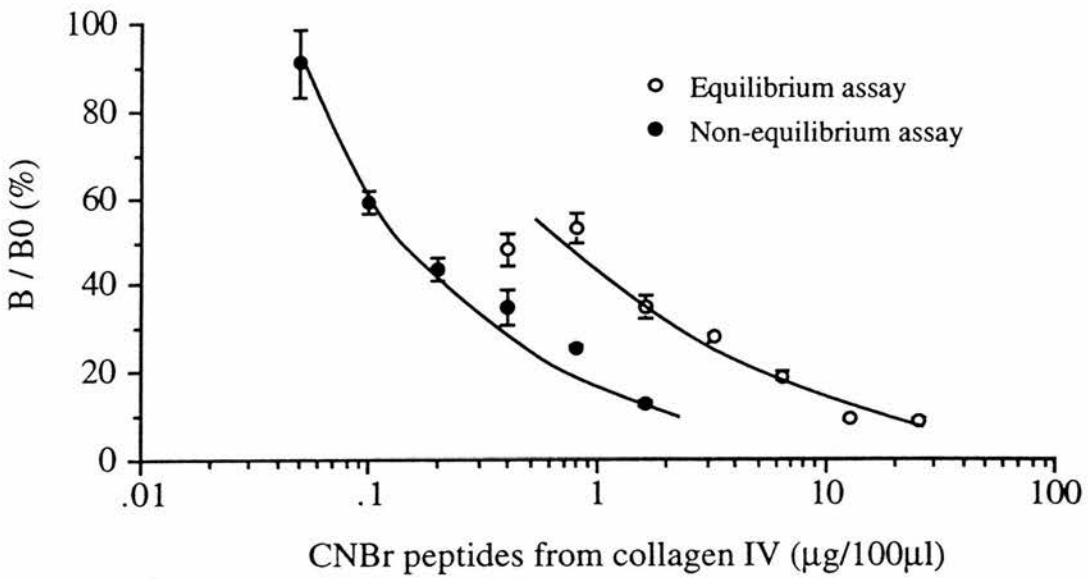


Figure 3.10 Comparison of an equilibrium versus a non-equilibrium ELISA for CNBr peptides of collagen IV. Each point on the graph represents the mean of three determinations ± SEM. B/B0% = amount of HRP-coupled second antibody which is bound, expressed as a percentage of that bound in the absence of soluble antigen.

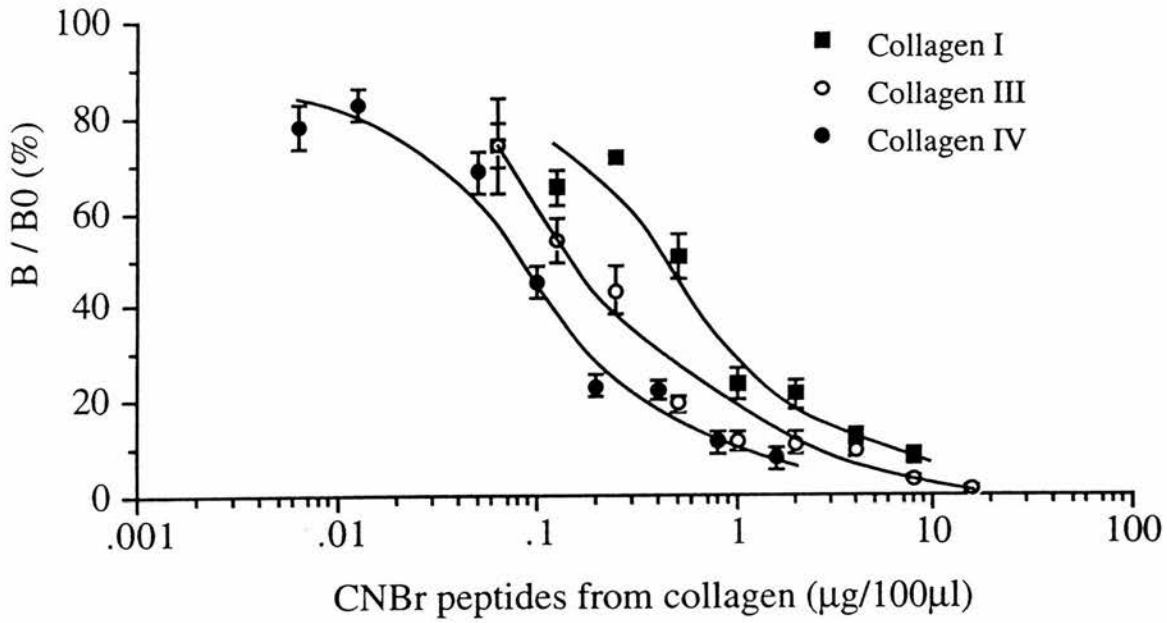


Figure 3.11 Cross-reaction of CNBr peptides from collagen I and collagen III with anti-collagen IV antiserum in an ELISA for collagen IV. Each point on the graph represents the mean of three determinations ± SEM. B/B0% = amount of HRP-coupled second antibody which is bound, expressed as a percentage of that bound in the absence of soluble antigen.

3.4.3 The Use of Monoclonal Antibodies.

In order to overcome the problem of lack of specificity of the polyclonal antisera, two commercially available monoclonal antisera were tested in a sandwich type assay using a polyclonal antiserum as the "capture" antiserum and a combination of the two monoclonal antibodies as the "conjugate" antibody. Normally the HRP is "conjugated" directly to the primary antibody. However, for the purposes of this assay, HRP conjugated anti-mouse antiserum was used. It was found, however, that optimisation of capture and conjugate antisera dilution was impossible due to a lack of antibody-antigen reaction (data not shown), even at a low antisera dilution (1 : 50).

In order to test the reactivity of both polyclonal antisera and monoclonal antibodies, microtitre plate wells were coated with collagen IV CNBr peptides (7.3 µg/well) as described in section 2.2.5.4. To half the wells was added a polyclonal antiserum (1 : 100 dilution) and to the remaining wells was added a 1 : 100 dilution of a 1 : 1 combination of the two monoclonal antibodies. Reactivity was measured with the use of HRP-coupled antispecies antisera and HRP substrate (section 2.2.5.4). It was found that while the polyclonal antiserum showed good reactivity as expected, the monoclonal antibodies showed no reaction with the peptides. The results are summarised in Table 3.2. The reactivity of HRP-coupled anti-mouse antiserum was tested by coating wells with a 1 : 100 dilution of a 1 : 1 combination of the two monoclonal antibodies followed by the addition of HRP-coupled anti-mouse antiserum. After incubation the wells were washed and substrate added. The results are summarised in Table 3.3 and show that HRP-coupled anti-mouse antiserum reacts well with the adsorbed monoclonal antibodies. The lack of any colour production by monoclonal antibodies was therefore due to their inability to bind to CNBr peptides of collagen IV.

3.4.4 Purity of Collagens I, III and IV for use in ELISA.

In order to assess the purity of human collagens I, III and IV from the Sigma Chemical Co., with respect to each other, CNBr digests of each collagen type were analysed by discontinuous SDS-PAGE (section 2.2.5.3). The patterns produced by each type are shown in Figure 3.12. Characteristic bands for collagens I and III were identified with reference to the literature (Laurent *et al.*, 1981a; Kelley *et al.*, 1984; Kirk *et al.*, 1984a; Bateman *et al.*, 1986) and demonstrated that these collagen types were distinct from each other. The patterns given by CNBr digested collagens III and IV were very similar demonstrating possible cross-contamination. The CNBr pattern produced by either type, however, did not conform to the patterns produced by purified rabbit collagens III and IV (Laurent *et al.*, 1981a), although both the Sigma

collagen types did possess a band which ran to the α1(III)-CB-5 position. Laurent *et al.* (1981a) demonstrated that collagen IV does not possess a peptide that runs to this position. The possibility that non-collagen proteins were contributing to the complexity of the patterns seen in the present study cannot be excluded. Interpretation of the gel might have been made easier had the samples not been reduced prior to electrophoresis. As previously mentioned in section 3.3, α1(III)-CB-9 may contaminate the α1(III)-CB-5 band when samples are reduced prior to electrophoresis (G.J. Laurent, personal communication). Running samples unreduced would, therefore, have allowed α1(III)-CB-5 to be measured more accurately, as α1(III)-CB-9 would have run as a disulphide bonded dimer and so, much more slowly. It was therefore impossible to draw any conclusions from the cross-reactivity experiments with respect to measuring collagen IV in lung.

In the lung parenchyma, collagens I and III are present in approximately 12 x and 6 x greater quantities, respectively, compared to collagen IV (Seyer *et al.*, 1976; Rennard *et al.*, 1980a; Madri and Furthmayr, 1980; Rennard and Crystal, 1982; Kirk *et al.*, 1984a). The quantities of collagens I and III in the lung compared to collagen IV may not necessarily be high enough to cross-react significantly with the anti-collagen IV antisera tested and therefore may not present a problem in an ELISA for collagen IV in human lung. Further experiments are required using pure collagens III and IV to establish accurate values for the cross-reactivity of anti-collagen IV antisera to collagens I and III.

Table 3.1. Cross-reaction of CNBr peptides of collagen I and collagen III with a number of anti-collagen IV antisera.

Antiserum	**Cross-reactivity (%)**	
	Collagen I	Collagen III
V.Duance	10.5	36.6
Euro-diagnostics BV.	5.1	14.5
Immunoaffinity purified Euro-diagnostics BV.	7.1	9.5
Southern Biotechnology	6.1	91.0

Cross-reaction was quantified at the 50% inhibitory level (Abraham, 1969).

Table 3.2. Reactivity of rabbit and mouse anti-collagen IV antisera with collagen IV CNBr peptide-coated micro-titre plate wells (7.3 μg/well).

Antiserum	**Mean Absorbance at 490 nm ± Standard Error of the Mean (SEM)**
Rabbit anti-collagen IV	1.46 ± 0.03
Mouse anti-collagen IV	ND

Anti-sera were diluted 1:100. ND = not detected. n = 6.

Table 3.3. Reactivity of HRP coupled anti-rabbit and anti-mouse antisera with rabbit and mouse anti-collagen IV antisera-coated microtitre plate wells (1:100 antiserum dilution/well).

Antiserum	**Mean Absorbance at 490 nm ± Standard Error of the Mean (SEM)**
Rabbit anti-collagen IV	1.59 ± 0.03
Mouse anti-collagen IV	1.04 ± 0.07

Anti-species antisera were diluted 1:500. n = 6.

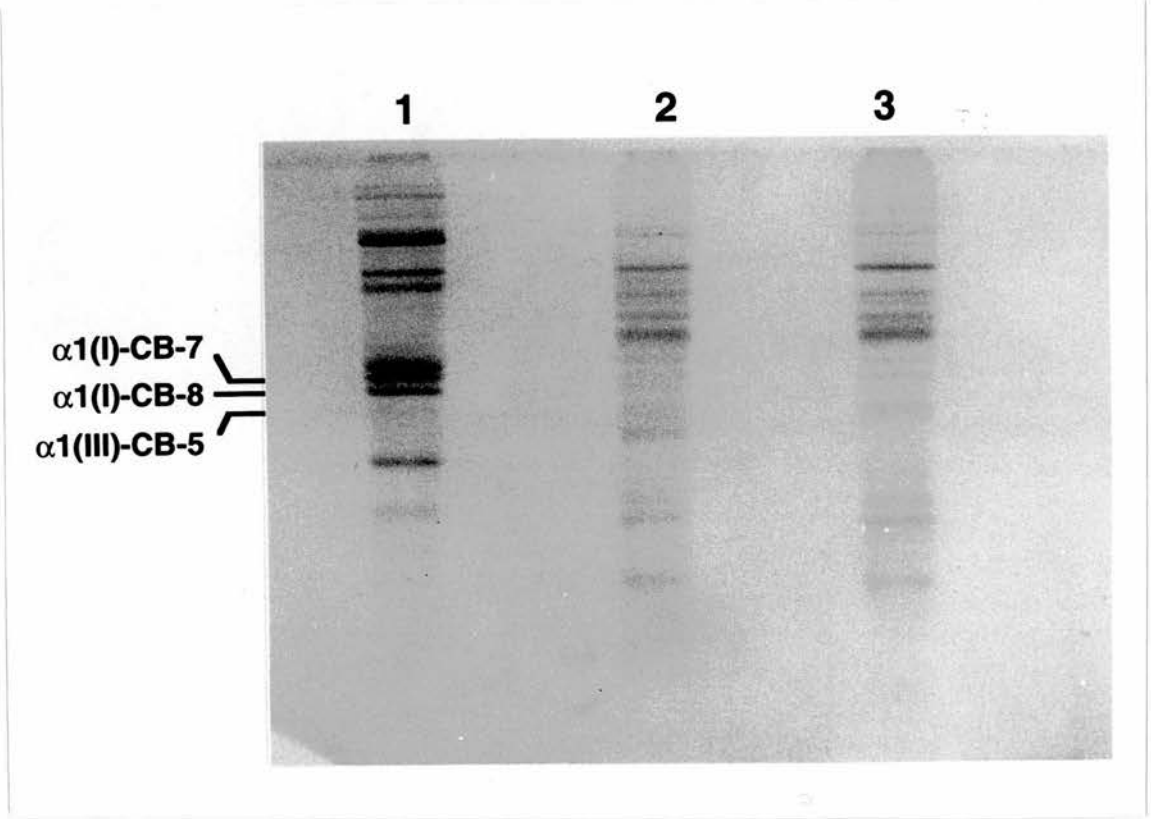


Figure 3.12 Discontinuous SDS-PAGE of CNBr digests of human collagens I, III and IV supplied by the Sigma Chemical Co. Track 1, collagen I; Track 2, collagen III; Track 3, collagen IV. Samples were reduced prior to electrophoresis.

CHAPTER 4. RESULTS

4.1 Assessment of Biochemical Techniques.

It was important to determine the reproducibility of the various biochemical techniques and the linearity of the calibration curves over the concentration ranges used in each assay.

4.1.1 Hydroxyproline Determination.

Hyp has been shown to be stable during hydrolysis in 6N HCl even in the presence of carbohydrates (Jackson and Cleary, 1967).

The within-batch imprecision of Hyp determination on the amino acid analyser (the PTC derivatisation technique) was assessed by determining the Hyp content of several aliquots from a single sample hydrolysate within a single batch. The between-batch imprecision of Hyp determination on the amino acid analyser was assessed by the repeated measurement of aliquots from a single sample hydrolysate in different batches. Different sample hydrolysates were used for assessing within-batch and between-batch imprecision. The results are summarised in Table 4.1.

The within-batch and between-batch imprecision of Hyp determination by HPLC with fluorimetric detection (the FMOC-Cl derivatisation technique) was assessed using paired data obtained from several sample hydrolysates analysed in duplicate both within a batch and between two batches (Percy-Robb *et al.*, 1980). The results are summarised in Table 4.1. The coefficients of variation are lower using the FMOC-Cl derivatisation technique compared with the PTC derivatisation technique and therefore the former was the method of choice.

The FMOC-Cl derivatisation technique was introduced somewhat later in the project and approximately 60% of Hyp measurements on agarose inflated lungs (64 samples) were made using the PTC derivatisation technique. In order to determine whether Hyp measurements from the two methods could be combined, results were obtained by each method on 10 different samples. Comparison by the Mann-Whitney rank sum test showed that the results obtained by either method did not differ significantly ($p=0.97$), and therefore it was concluded that Hyp results from both methods could be combined.

The calibration curves in which known amounts of pure Hyp standards were plotted versus the ratio of the peak area of Hyp to the peak area of a fixed amount of 3,4-DHP, were linear with correlation coefficients of 0.999 or greater over the concentration range 3.75 pmol-60.0 pmol Hyp/20 µl injected.

Table 4.1. Within-batch and between-batch variation in the quantitation of Hyp on the amino acid analyser (PTC-Hyp) and by HPLC with fluorimetric detection (FMOC-Hyp) and in the quantitation of isodesmosine. Hyp and isodesmosine were determined on known volumes of agarose inflated lung samples.

	Coefficient of variation (%)		
	PTC-Hyp	**FMOC-Hyp**	**isodesmosine**
Within-batch	13.9 n = 17	6.3 n = 11	9.0 n = 76
Between-batch	9.3 n = 7	4.3 n = 28	9.7 n = 14

PTC-Hyp = phenylthiocarbamyl-Hyp; FMOC-Hyp = 9-fluorenylmethylchloroformate-Hyp; n = number of observations.

4.1.2 Desmosine and Isodesmosine Determination by HPLC.

The within-batch and between-batch imprecision of desmosine and isodesmosine quantitation by HPLC was calculated using paired data obtained from several sample hydrolysates analysed in duplicate both within a batch and between two batches (Percy-Robb *et al.*, 1980). Results for isodesmosine are summarised in Table 4.1.

The calibration curves in which known amounts of desmosine or isodesmosine were plotted versus peak area, were linear with correlation coefficients of 0.999 or greater over the concentration range 0.0625 nmol-1.0 nmol/10 µl injected.

4.2 Regional Variation Within a Lung.

It was also important to assess the regional variation in AWUV, collagen, and elastin within a lung in order to determine whether samples from a single block of tissue were representative of the lung as a whole.

Table 4.2 shows mean values obtained for AWUV, Hyp and isodesmosine from the upper and lower lobes of two post mortem lungs after agarose inflation. There were no significant differences in AWUV ($p = 0.50$), Hyp per mm^3 ($p = 0.95$), or isodesmosine per mm^3 ($p = 0.40$) between the upper and lower lobes of the lung obtained from a 75 yr old smoker. Similarly, there were no significant differences in these parameters between the upper and lower lobes of the lung obtained from an 85 yr old non-smoker (AWUV, $p = 0.55$; Hyp, $p = 0.27$; isodesmosine, $p = 0.37$).

4.3 Analysis of Agarose Inflated Lungs.

HPLC determination of isodesmosine and desmosine on 102 parenchymal samples from nine agarose inflated lungs demonstrated that these elastin markers were present in near equimolar amounts over the concentration range measured (Figure 4.1; $r = 0.979$, $p < 0.001$). In view of the highly significant correlation between isodesmosine and desmosine, data for desmosine measurements were omitted from some of the comparisons (Tables 4.1, 4.2, 4.6 and 4.7). When the results for Hyp determination were plotted against those for isodesmosine a significant correlation was observed (Figure 4.2; $r = 0.863$, $p < 0.001$), thus demonstrating the degree to which collagen and elastin are co-distributed within the alveolar tissue at the 50 mm^3 level.

When the amounts of Hyp and isodesmosine in the 102 lung samples were expressed per mm^3 and plotted against their corresponding AWUV values, significant negative correlations were observed (Figure 4.3) such that as AWUV decreased (i.e. a decrease in the surface area of alveolar wall per unit volume), there was an increase in

Table 4.2. Anatomical variation in AWUV, collagen (determined as Hyp) and elastin (determined as isodesmosine) between the upper and lower lobes of one lung from a 75 year old smoker and one lung from an 85 year old non-smoker. The means and standard deviations (SD) were calculated from 12 samples per lobe.

	75 year old smoker			85 year old non-smoker		
Lobe	**AWUV (mm^2/mm^3)**	**Hyp ($pmol/mm^3$)**	**Isodesmosine ($pmol/mm^3$)**	**AWUV (mm^2/mm^3)**	**Hyp ($pmol/mm^3$)**	**Isodesmosine ($pmol/mm^3$)**
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Upper	18.61 (1.85)	7080 (2500)	27.7 (4.8)	14.74 (1.04)	6440 (640)	25.9 (6.7)
Lower	17.86 (2.64)	7150 (3450)	30.2 (10.7)	15.23 (1.58)	5876 (1270)	28.0 (4.2)

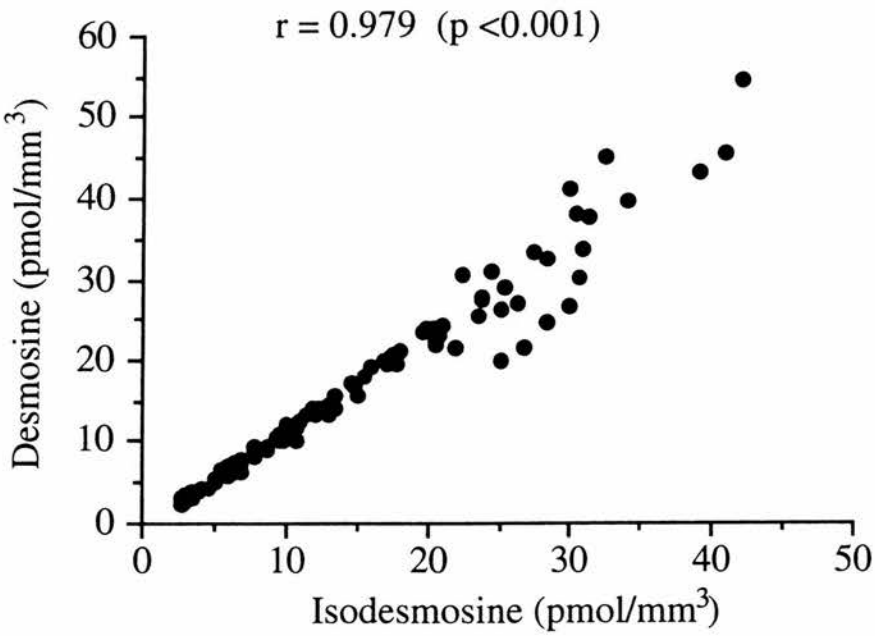


Figure 4.1 Relationship between the two elastin specific cross-linking amino acids, desmosine and isodesmosine, over the concentration range measured. The results were obtained from 102 samples from nine lungs.

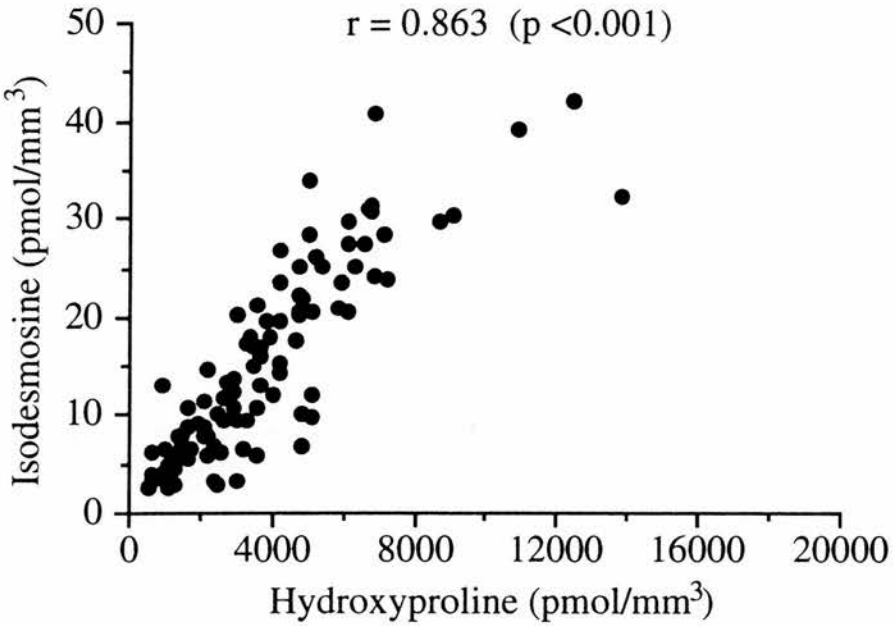


Figure 4.2 Relationship between collagen (determined as hydroxyproline) and elastin (determined as isodesmosine) within 102 samples from nine lungs.

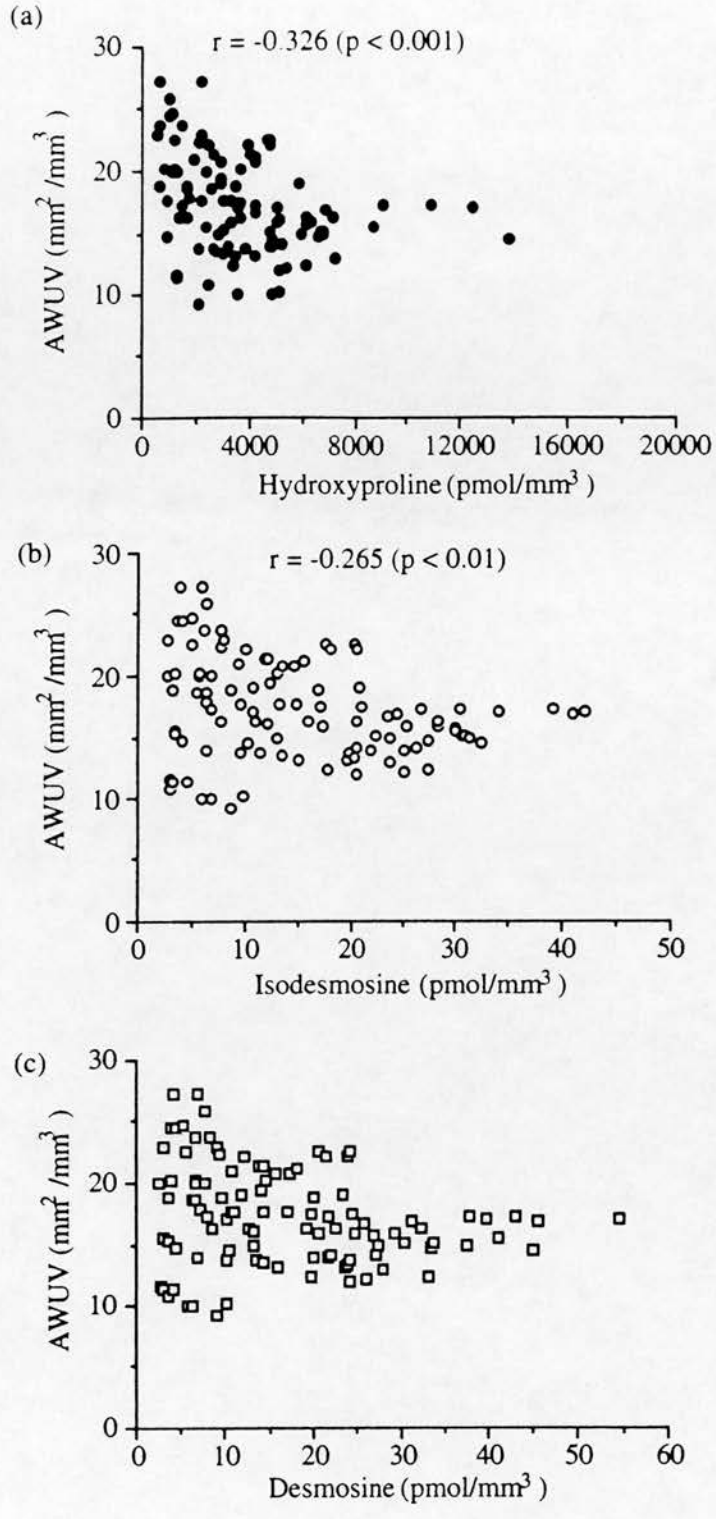


Figure 4.3 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^3 and the alveolar wall surface area per unit volume (AWUV) for 102 samples taken from nine smokers' lungs without macroscopic emphysema.

the amount of Hyp ($r = -0.326$, $p < 0.001$) and isodesmosine ($r = -0.265$, $p < 0.01$). The correlation between desmosine content per mm^3 and AWUV was not significant ($r = -0.237$; Figure 4.3).

Stronger negative correlations were found when the Hyp and isodesmosine data were normalised to take into account surface area of alveolar wall, by dividing the mm^3 data by their corresponding AWUV values to give amounts of Hyp and isodesmosine per mm^2 of alveolar wall. The results are summarised in Figure 4.4 and show highly significant negative correlations between AWUV and Hyp per mm^2 ($r = -0.530$, $p < 0.001$), and between AWUV and isodesmosine per mm^2 ($r = -0.458$, $p < 0.001$). A highly significant negative correlation was also found between desmosine content per mm^2 and AWUV ($r = -0.422$, $p < 0.001$; Figure 4.4). The data show that as the surface area of alveolar wall per unit volume decreases there is an increase in the amount of collagen and elastin in the alveolar wall that remains.

The relationships between AWUV and Hyp, and between AWUV and isodesmosine or desmosine, within each of the two post mortem lungs are shown in Figures 4.5 - 4.8. A significant negative correlation between AWUV and Hyp, and between AWUV and isodesmosine or desmosine was observed only in the lung of the 75 yr old smoker and only when the biochemical data were expressed per mm^2.

4.4 Analysis of Formalin Inflated Lungs.

In order to increase the number of cases to include those with different forms of emphysema, a store of formalin inflated lungs from the Department of Pathology, University of Edinburgh, were analysed (section 2.1.2 and section 2.1.4.2). Tissue samples were analysed biochemically (and for adjacent slice AWUV) from areas remote from macroscopic emphysematous lesions. Of the 29 smokers' lungs included in this part of the study, eight had case AWUV values below the lower limit of "normality" for non-smokers (Gillooly and Lamb, 1993a). Of these lungs, five had panacinar emphysema, one had centriacinar emphysema and two had a mixture of centriacinar and panacinar emphysema (Figure 4.9).

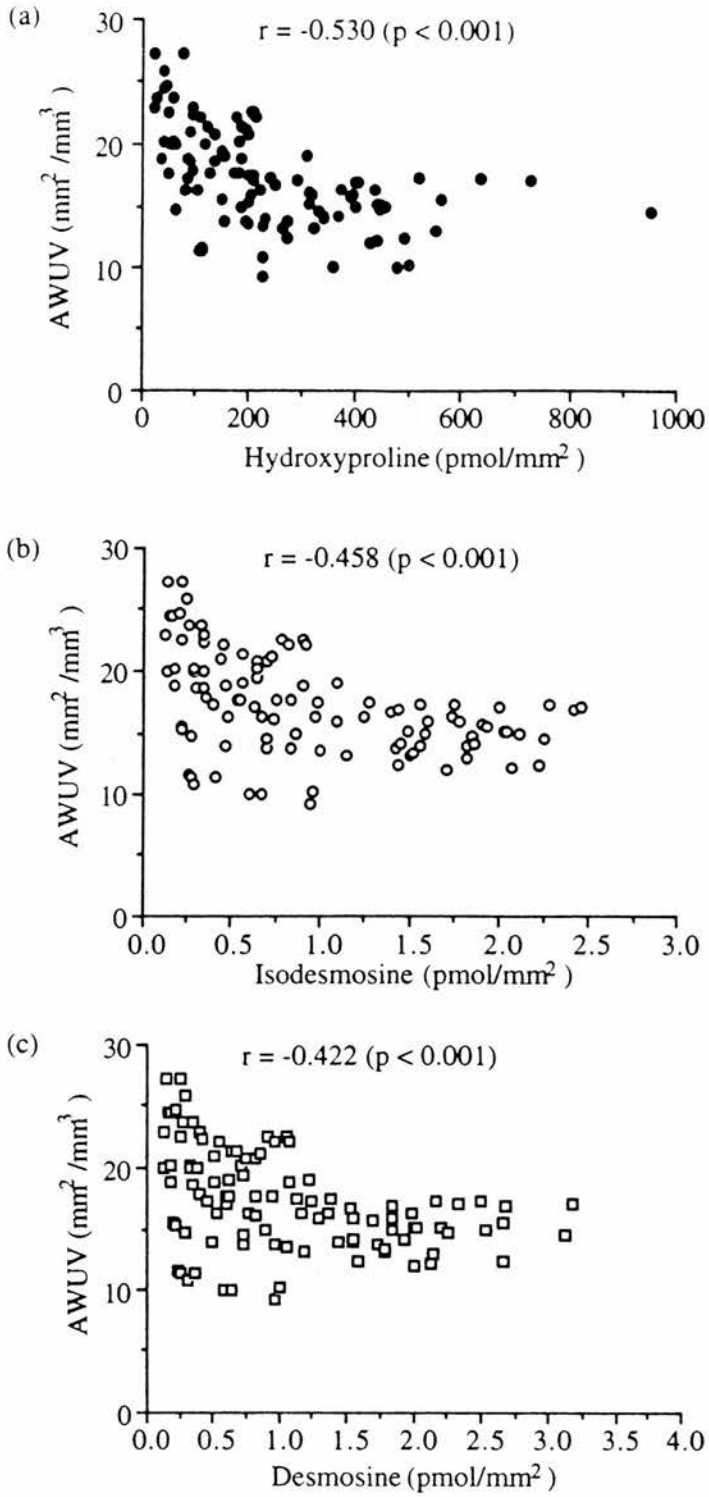


Figure 4.4 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^2 and the alveolar wall surface area per unit volume (AWUV) for 102 samples taken from nine smokers' lungs without macroscopic emphysema.

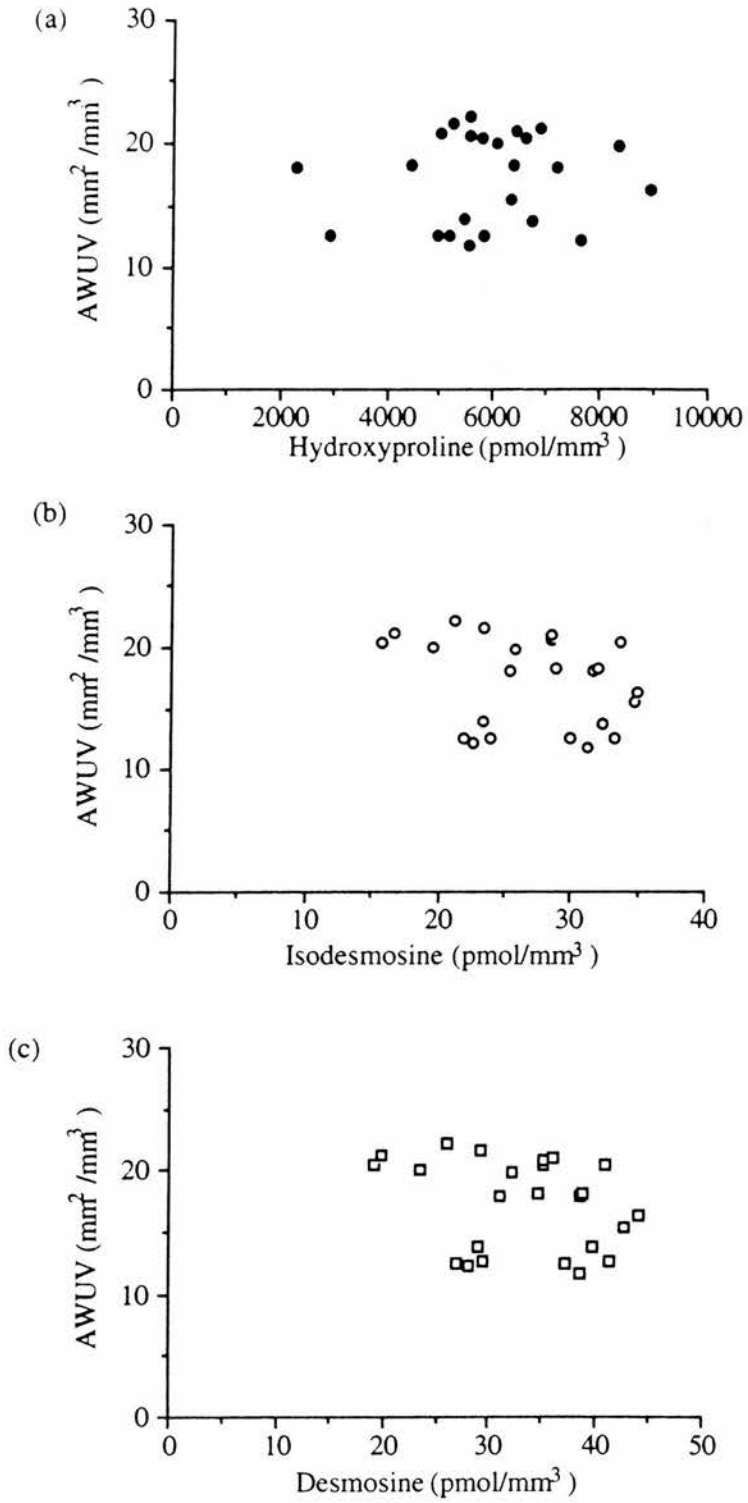


Figure 4.5 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^3 and the alveolar wall surface area per unit volume (AWUV) of 24 samples taken from the upper and lower lobes of a single lung from a 75 year old smoker with no signs of macroscopic emphysema.

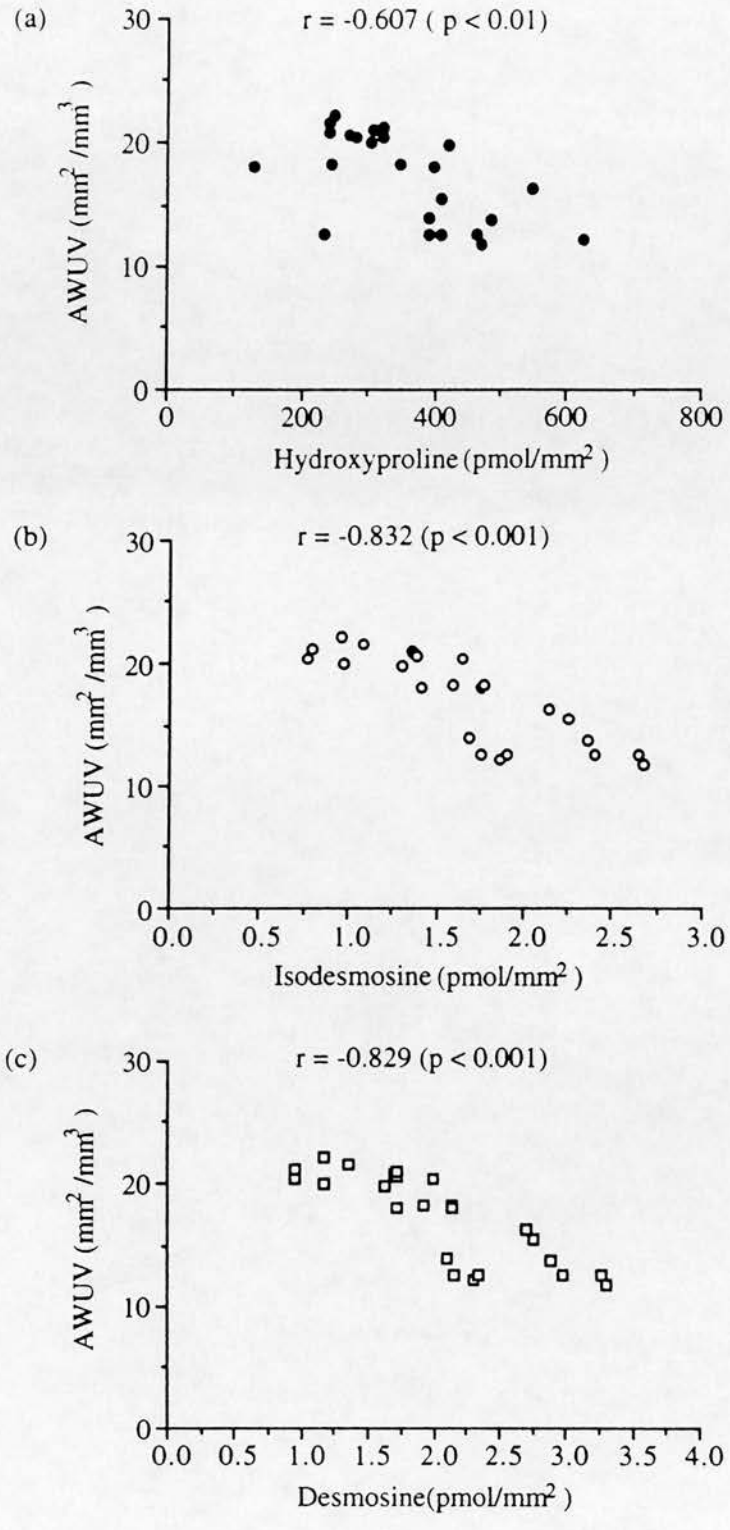


Figure 4.6 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^2 and the alveolar wall surface area per unit volume (AWUV) of 24 samples taken from the upper and lower lobes of a single lung from a 75 year old smoker with no signs of macroscopic emphysema.

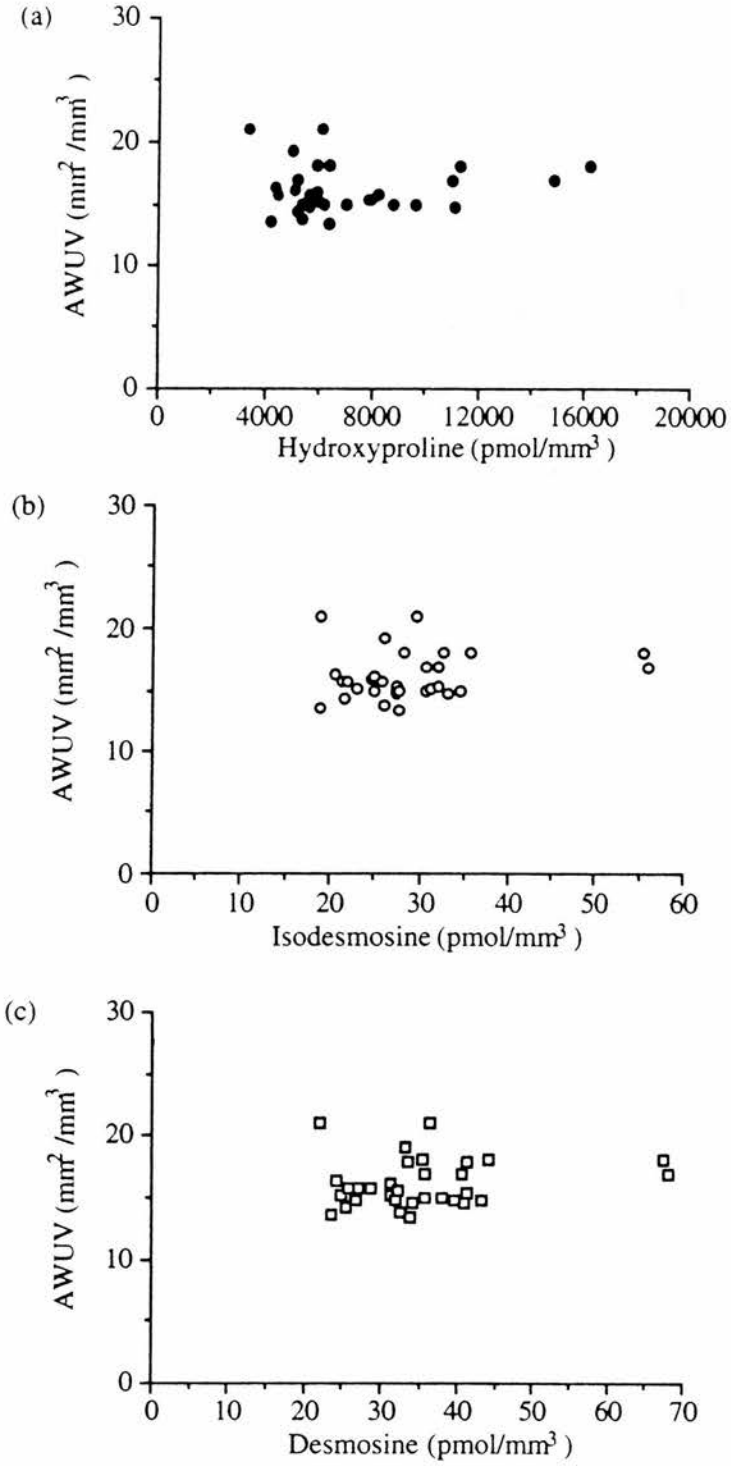


Figure 4.7 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^3 and the alveolar wall surface area per unit volume (AWUV) of 32 samples taken from the upper and lower lobes of a single lung from an 85 year old non-smoker.

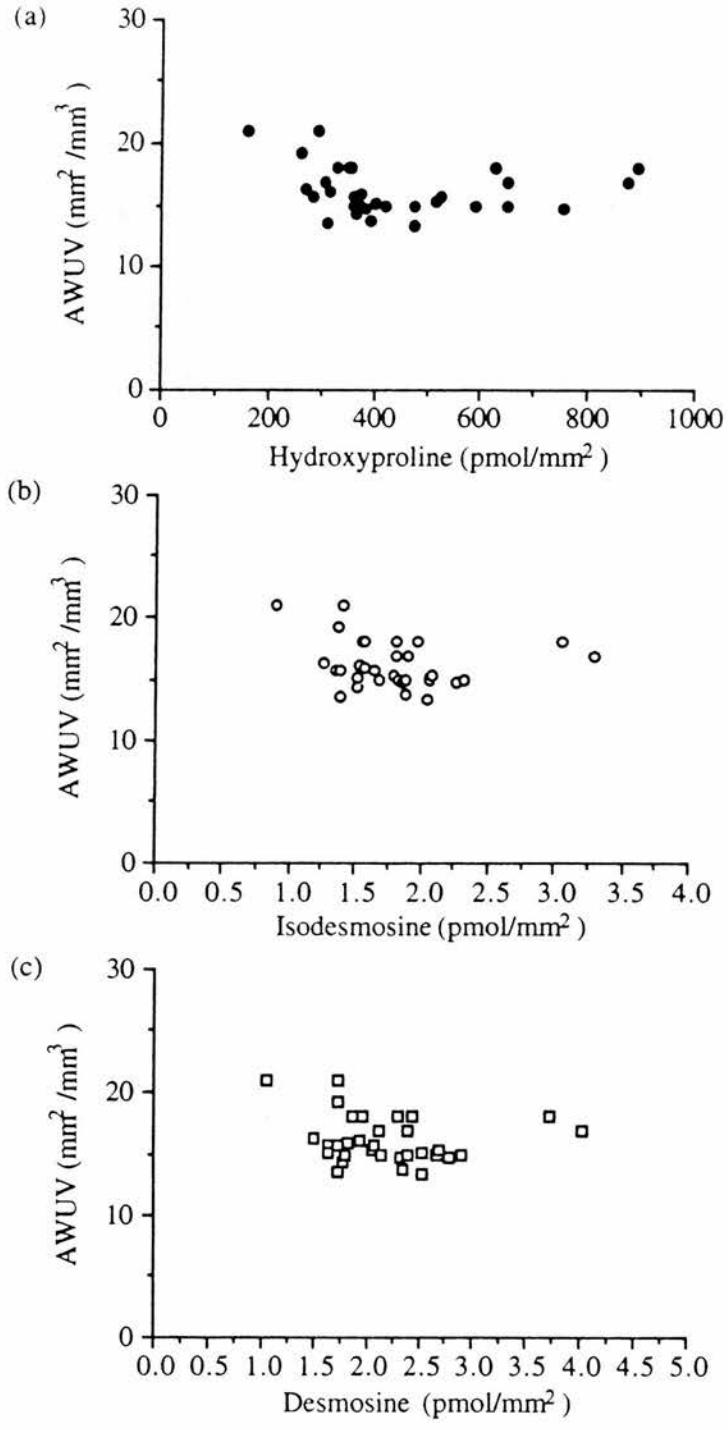


Figure 4.8 Relationship between (a) the hydroxyproline, (b) the isodesmosine and (c) the desmosine content per mm^2 and the alveolar wall surface area per unit volume (AWUV) of 32 samples taken from the upper and lower lobes of a single lung from an 85 year old non-smoker.

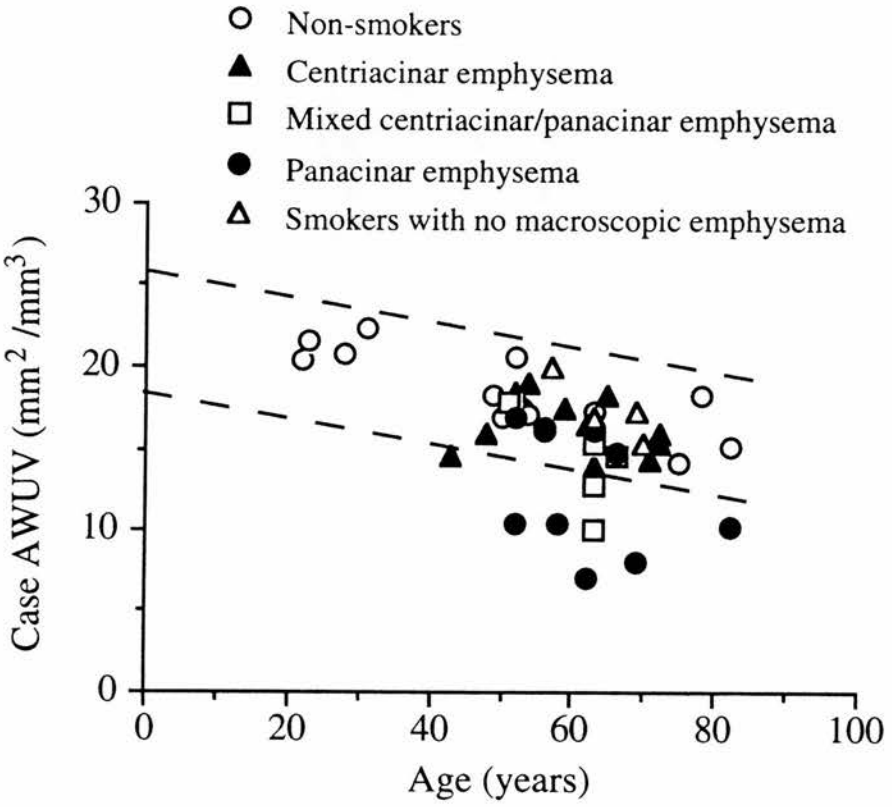


Figure 4.9 Case AWUV plotted against age in a sample of non-smokers and smokers showing eight subjects with case AWUV values falling below the lower limit of "normality" for non-smokers.

In order to determine the effect of formalin fixation on the determination of Hyp, 12 samples from different regions of the same lung were taken and half fixed in 10% formalin for one week. The mean value for Hyp determined by the FMOC-Cl derivatisation technique for the unfixed samples was 6.6 nmol/mm^3 (SD 2.8 nmol/mm^3) and the mean value for Hyp for the fixed samples was 8.8 nmol/mm^3 (SD 2.6 nmol/mm^3). By the Mann-Whitney rank sum test there was no significant difference between the two groups with respect to Hyp content per mm^3. Furthermore, the storage times of the emphysematous and non-emphysematous groups were similar and therefore any possible effects of long term storage in formalin would apply equally to all groups.

Hyp data were again expressed per mm^3 and per mm^2. In order to obtain an accurate measurement of surface area with which to normalise the Hyp/mm^3 data, AWUV values were determined on an immediately adjacent slice to the pooled slices used for Hyp determination (section 2.1.4.2).

4.4.1 Analysis of Non-smokers' Lungs.

Figure 4.10 shows the relationship between age and AWUV in the group of non-smokers. There was a significant decrease in case AWUV with increasing age ($r = -0.808$, $p < 0.001$) similar to that seen in a study of a larger number of non-smokers' lungs (Gillooly and Lamb, 1993a).

There was no significant correlation between case AWUV and Hyp content either per mm^3 or per mm^2 (Figure 4.11). Similarly, there was no significant correlation between age and Hyp content per mm^3 or per mm^2 (Figure 4.12), though the trend in Figure 4.12 (b) is consistent with the age-related loss in AWUV shown in Figure 4.10.

4.4.2 Analysis of Smokers' Lungs.

The age ranges of the smoking and the non-smoking groups are summarised in Table 4.3. The median and mean (±SD) age, case AWUV, Hyp/mm^3 and Hyp/mm^2 data for the all groups are summarised in Table 4.4. The four youngest non-smokers have been excluded in order to minimise the effect of age on the morphological and biochemical data between smokers and non-smokers. Thus, the mean age for the non-smoking group was 61.2 (SD 12.5), and the mean age for the smoking group was 61.6 (SD 8.5). Comparison by the Mann-Whitney rank sum test showed that there was no significant difference between non-smokers and smokers with respect to age.

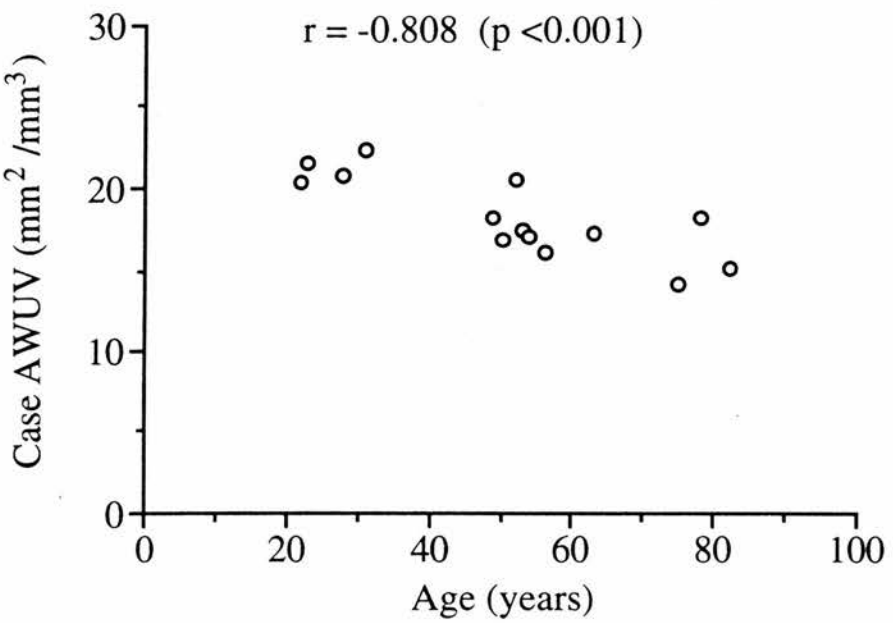


Figure 4.10 Relationship between case AWUV and age in a sample of 14 non-smokers' lungs.

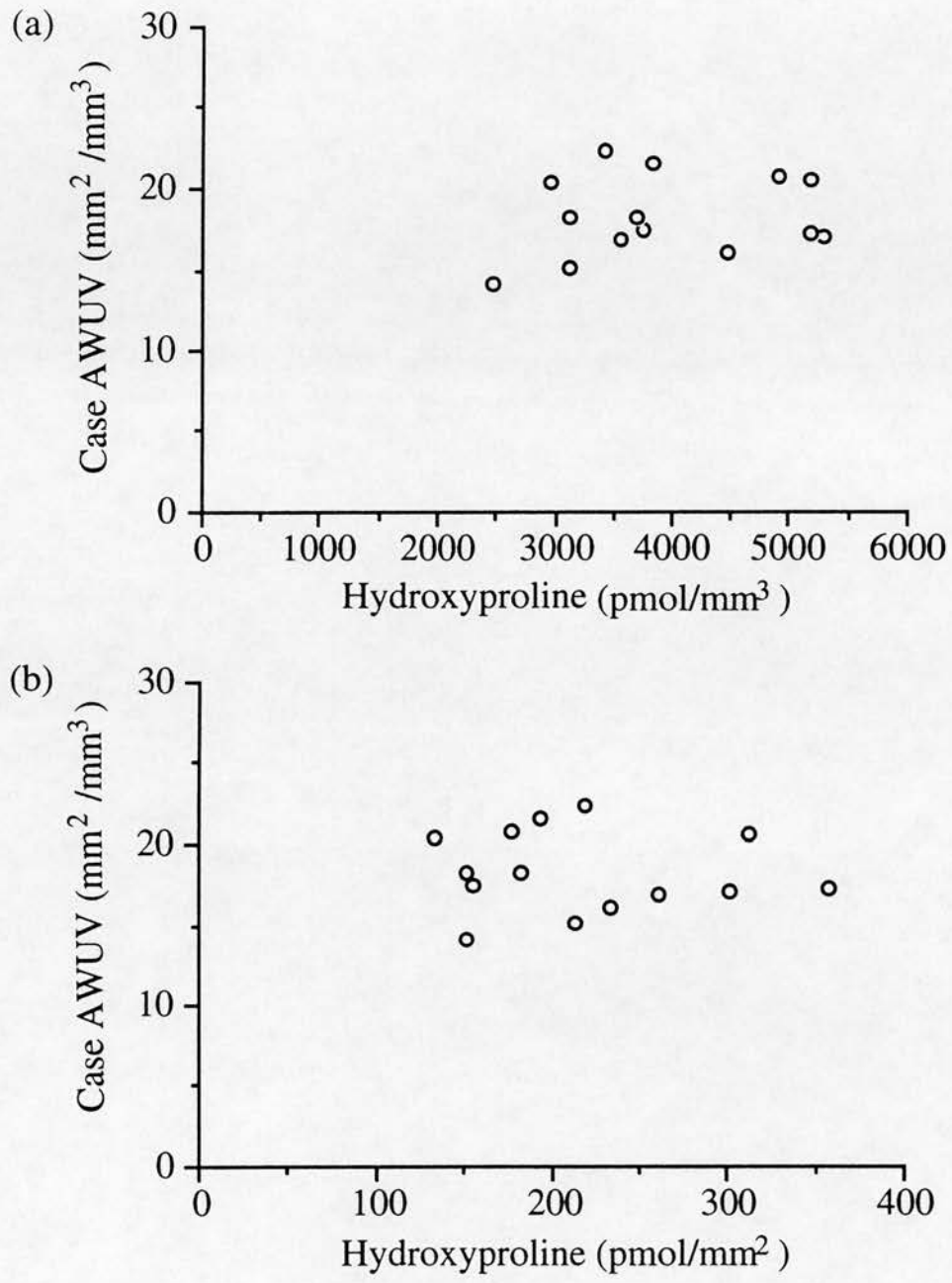


Figure 4.11 Case AWUV plotted against (a) hydroxyproline content per mm^3 and (b) hydroxyproline content per mm^2 for single samples from each of 14 non-smokers' lungs. Hydroxyproline values per mm^3 were converted to hydroxyproline per mm^2 by dividing the former figure by the AWUV value determined on the adjacent tissue slice to those pooled for hydroxyproline determination.

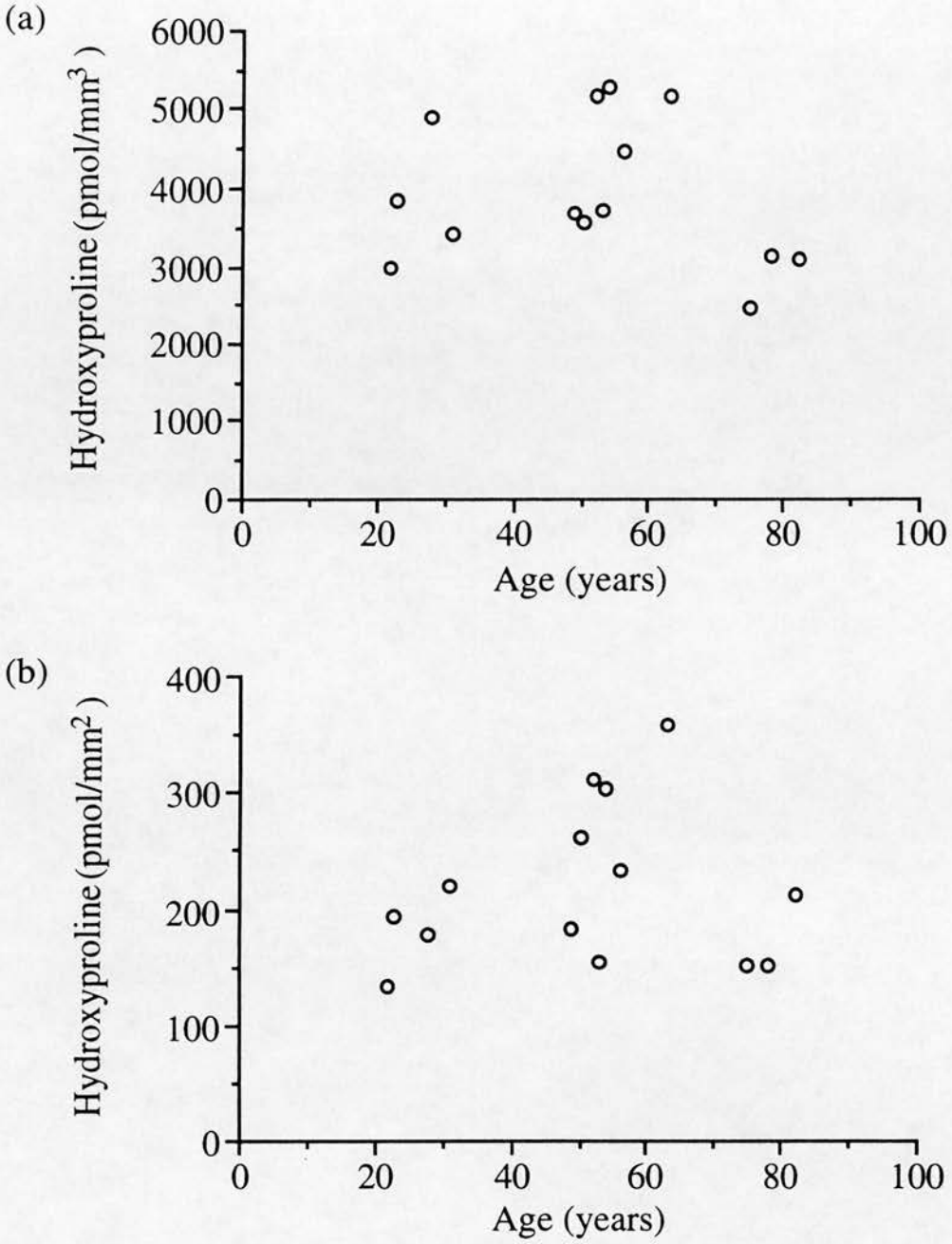


Figure 4.12 Age plotted against (a) hydroxyproline content per mm^3 and (b) hydroxyproline content per mm^2 for single samples from each of 14 non-smokers' lungs. Hydroxyproline values per mm^3 were converted to hydroxyproline per mm^2 by dividing the former figure by the AWUV value determined on the adjacent tissue slice to those pooled for hydroxyproline determination.

Table 4.3 . Smoking histories and age ranges of the formalin inflated cases.

Smoking History	Age Range (years)	n
Non-smokers	22 - 31	4
	49 - 82	10
Smokers (all data)	43 - 82	29
CAE	43 - 72	11
PAE	52 - 82	9
CAE/PAE	51 - 66	5
No macroscopic emphysema	57 - 70	4

CAE = centriacinar emphysema; PAE = panacinar emphysema;
CAE / PAE = mixed centriacinar / panacinar emphysema;
n = number of subjects per group.

Table 4.4 Descriptive statistics for age, AWUV and Hyp content of the formalin inflated cases. Significance values were calculated from medians using the Mann-Whitney rank sum test. The four youngest non-smokers were excluded from the statistical analyses.

Smoking History	n	Age (years)			AWUV (mm^2/mm^3)			Hyp ($pmol/mm^3$)			Hyp ($pmol/mm^2$)		
		Median	Mean	(SD)	Median	Mean	(SD)	Median	Mean	(SD)	Median	Mean	(SD)
Non-smokers	10	55.0	61.2	(12.5)	17.18	17.07	(1.81)	3720	3980	(1000)	223.0	232.0	(74.2)
Smokers (all data)	29	63.0	61.6	(8.5)	15.38	14.80	(3.28)	5350 *	6540	(3250)	457.0 **	498.1	(327.0)
CAE	11	62.0	60.1	(9.9)	15.91	16.32	(1.75)	5010 *	7100	(3990)	333.0 *	394.3	(149.0)
PAE	9	62.0	62.2	(9.5)	10.45 *	12.23	(3.74)	6540 *	7170	(3310)	461.0 **	643.4	(505.7)
CAE/PAE	5	63.0	61.2	(5.8)	14.42	14.07	(2.91)	4740	4990	(1970)	494.0 **	588.6	(243.3)
No macroscopic emphysema	4	66.0	64.5	(6.0)	17.01	17.30	(1.93)	5830	5480	(1750)	319.5	343.5	(116.1)

* $p < 0.05$, ** $p < 0.005$ compared to the value for the non-smokers. CAE = centriacinar emphysema; PAE = panacinar emphysema; CAE/PAE = mixed centriacinar / panacinar emphysema; n = number of subjects per group.

Smokers as a whole had a mean case AWUV of 14.80 (SD 3.28) which was lower than the mean case AWUV for non-smokers (17.07, SD 1.81). However, comparison by the Mann-Whitney rank sum test showed that the difference was barely significant ($p = 0.05$).

The mean Hyp content for the non-smoking group was 3980 pmol/mm^3 (SD 1000 pmol/mm^3) and 232 pmol/mm^2 (SD 74.2 pmol/mm^2). The mean Hyp content for the smoking group was 6540 pmol/mm^3 (SD 3250 pmol/mm^3) and 498.1 pmol/mm^2 (SD 327.0 pmol/mm^2). Comparison by the Mann-Whitney rank sum test showed Hyp/mm^3 was significantly higher in the smoking group than in the non-smokers ($p < 0.05$). The significance was greater ($p < 0.005$) when Hyp/mm^3 was normalised to take into account slice surface area and expressed per mm^2.

Of the smoking group, lungs with centriacinar emphysema had a mean Hyp content of 7100 pmol/mm^3 (SD 3990 pmol/mm^3) and 394.3 pmol/mm^2 (SD 149.0 pmol/mm^2). Lungs with panacinar emphysema had a mean Hyp content of 7170 pmol/mm^3 (SD 3310 pmol/mm^3) and 643.4 pmol/mm^2 (SD 505.7 pmol/mm^2). Comparison by the Mann-Whitney rank sum test showed that smokers' lungs with centriacinar emphysema had a significantly higher content of Hyp than non-smokers lungs ($p < 0.05$). The level of significance remained the same whether Hyp was expressed per mm^3 or per mm^2. Similarly, smokers' lungs with panacinar emphysema had a significantly higher Hyp content than non-smokers lungs. When the biochemical data were expressed per mm^2, however, the level of significance increased ($p < 0.005$ compared to $p < 0.05$).

Smokers' lungs with no macroscopic emphysema had a mean Hyp content of 5480 pmol/mm^3 (SD 1750 pmol/mm^3) and 343.5 pmol/mm^2 (SD 116.1 pmol/mm^2). Though higher than in non-smokers, the Hyp content of the smokers' lungs, whether expressed per mm^3 or per mm^2, was not significantly higher, by the Mann-Whitney rank sum test, than in non-smokers.

Smokers' lungs with a mixture of centriacinar and panacinar emphysema had a mean Hyp content of 4990 pmol/mm^3 (SD 1970 pmol/mm^3) and 588.6 pmol/mm^2 (SD 245.3 pmol/mm^2). Comparison by the Mann-Whitney rank sum test showed that there was no statistically significant difference between this group and the non-smoking group with respect to Hyp content per mm^3. However, when Hyp was normalised to take into account slice surface area per unit volume and expressed per mm^2, comparison by the Mann-Whitney rank sum test showed that the smokers'

lungs with a mixture of centriacinar emphysema and panacinar emphysema had a significantly higher Hyp content per mm^2 than the non-smokers ($p < 0.005$).

There was no statistically significant difference between smoking groups with respect to Hyp content whether Hyp was expressed per mm^3 or per mm^2.

In summary, compared to non-smokers, smokers were found to have a significantly higher collagen content in areas of lung free from macroscopic emphysema. Smokers' lungs which had lesions of macroscopic centriacinar emphysema and macroscopic panacinar emphysema present were found to have the highest collagen content in areas of lung free from macroscopic emphysema, compared to non-smokers.

4.5 Analysis of Lungs from α1-Protease Inhibitor (α1-Pi) Deficient Subjects.

Lungs from three subjects with α1-Pi deficiency, and from two smokers and a non-smoker were analysed both morphometrically (for AWUV) and biochemically (for Hyp and isodesmosine). One lobe was inflated with 70% (v/v) ethanol to allow the determination of isodesmosine to be made as well as Hyp (section 2.1.4.3). Further Hyp measurements were made on the second lobe which was inflated with 10% buffered formalin (section 2.1.4.3).

4.5.1 Analysis of Formalin Inflated Lobes.

Hyp data were expressed per mm^3 and per mm^2, where the latter was derived by dividing the former figure by the adjacent slice AWUV (section 2.1.4.3). When samples from the non-smoker and the two smokers were compared, using the Mann-Whitney rank sum test, to the α1-Pi deficient group (Table 4.5), the α1-Pi group had a significantly lower AWUV ($p < 0.05$) and a significantly higher Hyp content per mm^3 and per mm^2 ($p < 0.005$). The smoking group was not significantly different from the non-smoker with respect to AWUV, Hyp/mm^3 or Hyp/mm^2.

4.5.2 Analysis of Ethanol Inflated Lobes.

Due to technical difficulties in inflating lung tissue with ethanol, AWUV measurements on these lobes were not possible. Isodesmosine content was therefore expressed per mm^3 and per mm^2 where the latter was derived by dividing the former by the mean AWUV which had been determined on the formalin inflated lobes. Hyp data was expressed per mm^3. When the individual cases were again grouped into a smoking group and an α1-Pi deficient group and compared, using the

Mann-Whitney rank sum test, to the non-smoker (Table 4.6), there were no significant differences between the α1-Pi group and the non-smoker with respect to isodesmosine content either per mm^3 or per mm^2. The smoking group did, however, have a significantly higher isodesmosine content per mm^3 and per mm^2 compared to the non-smoker ($p < 0.05$ and $p < 0.005$ respectively) and compared to the α1-Pi group ($p < 0.005$ and $p < 0.05$ respectively).

The mean Hyp content per mm^3 for all samples from the formalin inflated lobes was 5922 pmol/mm^3 (SD 2624 pmol/mm^3; n = 37) and for all samples from the ethanol inflated lobes the mean Hyp content per mm^3 was 6473 pmol/mm^3 (SD 2893 pmol/mm^3; n = 40). Comparison by the Mann-Whitney rank sum test showed that these two values did not differ significantly and therefore, despite problems inflating lobes with ethanol, it was felt that the isodesmosine and Hyp results from the ethanol inflated lobes accurately reflected changes between the lungs studied. These changes were reflected in the mean Hyp : isodesmosine ratios of the groups (Table 4.7). Compared to the non-smoker, the smoking group and the α1-Pi group had a significantly higher Hyp : isodesmosine ratio ($p < 0.05$ and $p < 0.005$ respectively). The α1-Pi group also had a significantly higher Hyp : isodesmosine ratio compared to the smoking group ($p < 0.005$). The increased Hyp : isodesmosine ratio of the smoking group compared to the non-smoker reflected a proportionally greater increase in collagen content compared to the increase in elastin content. The increased Hyp : isodesmosine ratio of the α1-Pi group compared to both the non-smoker and the smoking group reflected a significant increase in collagen content and no change in elastin content.

Because only one non-smoker was included in this part of the study the results should at this stage be regarded only as preliminary. In summary, compared to the non-smoker, the α1-Pi deficient lungs as a group were found to have a significantly higher collagen content per mm^3 of inflated lung and per mm^2 of alveolar wall and no loss of elastin. Compared to the non-smoker, the smokers' lungs as a group were found to have a significantly higher collagen and elastin content per mm^3 of inflated lung and per mm^2 of alveolar wall.

Table 4.5 Descriptive statistics for AWUV and Hyp content in formalin inflated lobes from the lungs of three subjects with α1-Pi deficiency, and from one non-smoker and two smokers. Significance values were calculated from medians using the Mann-Whitney rank sum test.

	n^a	n^b	AWUV (mm^2/mm^3)			Hyp ($pmol/mm^3$)			Hyp ($pmol/mm^2$)		
			Median	Mean	(SD)	Median	Mean	(SD)	Median	Mean	(SD)
α1-Pi	3	20	11.45	11.58	(3.91)	7203	7219	(2672)	539.5	705.3	(389.3)
Non-smoker	1	6	15.79 *	16.38	(2.64)	3203 **	3604	(1022)	208.0 **	221.2	(57.4)
Smokers	2	11	17.30 *	15.19	(3.31)	4465 *	4829	(1672)	273.6 **	331.7	(131.5)

* $p < 0.05$, ** $p < 0.005$ compared to the α1-Pi deficient lungs. There was no significant difference between the smokers and the non-smoker with respect to AWUV, Hyp/mm^3 and Hyp/mm^2. n^a = number of subjects; n^b = total number of samples.

Table 4.6 Descriptive statistics for isodesmosine content per mm^3 and per mm^2 in ethanol inflated lobes from the lungs of a single non-smoker, three subjects with α1-Pi deficiency and two smokers. Significance values were calculated from medians using the Mann-Whitney rank sum test.

	n^a	n^b	Isodesmosine ($pmol/mm^3$)			Isodesmosine ($pmol/mm^2$)		
			Median	Mean	(SD)	Median	Mean	(SD)
Non-smoker	1	6	17.4	19.9	(5.1)	1.06	1.22	(0.31)
α1-Pi	3	23	18.6	19.7	(6.5)	1.61	1.73	(0.62)
Smokers	2	11	32.3*	33.3	(11.8)	2.05 **	2.19	(0.61)

* $p < 0.05$, ** $p < 0.005$ compared to the non-smoker. The smokers' lungs also had a significantly higher isodesmosine content per mm^3 ($p < 0.005$) and per mm^2 ($p < 0.05$) compared to the α1-Pi deficient subjects. n^a = number of subjects; n^b = total number of samples.

Table 4.7. Descriptive statistics for the Hyp : isodesmosine ratio in ethanol inflated lobes from the lungs of a single non-smoker, three subjects with α1-Pi deficiency and two smokers. Ratios were calculated from Hyp and isodesmosine values per mm^3. Significance values were calculated from medians using the Mann-Whitney rank sum test.

	n^a	n^b	Hyp : isodesmosine ratio		
			Median	Mean	(SD)
Non-smoker	1	6	189.9	195.8	(21.5)
α1-Pi	3	23	331.6 **	319.4	(50.6)
Smokers	2	11	242.9 *	242.8	(26.7)

* $p < 0.05$, ** $p < 0.005$ compared to the non-smoker; the α1-Pi deficient lungs also had a significantly higher Hyp : isodesmosine ratio compared to the smokers' lungs ($p < 0.005$). n^a = number of subjects; n^b = total number of samples.

CHAPTER 5. DISCUSSION.

5.1 Analysis of Collagen and Elastin in Agarose Inflated Lung Samples.

Two of the major pitfalls in the analysis of lung connective tissue, the use of whole lungs (section 1.8) and the use of inappropriate modes of expressing the data (section 1.8), both of which have led to conflicting results, have been overcome in this study. First, pleura and major bronchovascular structures were excluded from the small pieces of tissue used in this study so that the biochemical and morphometric measurements were more representative of changes in the acinar unit itself. Second, samples of known volume were used and AWUV was determined on adjacent slices in order that the biochemical data could be expressed with reference to both a unit volume of inflated lung parenchyma (per mm^3), and to a unit area of alveolar wall (per mm^2), thus accurately taking into account differences in the amount of alveolar wall per unit volume from sample to sample.

In the analysis of the two post mortem lungs, one from a smoker and the other from a non-smoker, there were no significant differences between upper and lower lobes with respect to AWUV, Hyp/mm^3 and isodesmosine/mm^3 (section 4.2). Kirk *et al.* (1986) were also unable to detect a significant difference in collagen content between upper and lower lobes or vertically throughout the lung. Therefore, gravitational pull on the human lung would seem to have no significant effect on the vertical distribution of airspace size and collagen or elastin content. There may be hidden mechanisms, however, which have evolved to minimise the possible effects of gravity.

A strong correlation was observed when Hyp/mm^3 was plotted against isodesmosine/mm^3, thus demonstrating the close association of collagen and elastin within the acinar unit. These findings are consistent with those of Mercer and Crapo (1990), who used morphometric techniques to evaluate the spatial distribution of collagen and elastin fibres in rat and normal human lung parenchyma. They found that in normal human lung the amounts of collagen and elastin fibres were greatest in the walls of the alveolar ducts. Within the alveolar walls, the amounts of collagen and elastin were greatest within the first 20 μm of wall adjacent to the duct. The ratio of collagen fibres to elastin fibres remained remarkably constant throughout all the alveolar tissue. It is possible therefore that the wide range of values for Hyp/mm^3 and for isodesmosine/mm^3 observed in the present study may reflect not only the varying amounts of tissue present per mm^3 but also the portion of the acinar unit sampled.

One of the important and surprising findings of this study was that as AWUV decreased there was an increase in the amount of hydroxyproline, isodesmosine and desmosine, per unit volume of alveolar tissue. The resected lung samples showed no obvious signs of macroscopic emphysema, and the mean AWUV values for the agarose inflated zones were within the normal range for non-smokers (Gillooly and Lamb, 1993a). These results therefore, on essentially normal lungs, may simply reflect the heterogeneity within the acinar unit with respect to collagen, elastin and airspace size. Within normal lung, distal regions of the acinar unit, which consist mostly of alveoli, have the highest AWUV values and, according to Mercer and Crapo (1990), the lowest amount of collagen and elastin, at least compared to alveolar ducts. More proximal regions of the acinar unit, which consist of terminal bronchioles, respiratory bronchioles and alveolar ducts, have the lowest AWUV values but may contain higher amounts of collagen and elastin.

The negative correlation was stronger when the biochemical data were expressed per unit area of alveolar tissue. This is not surprising, since even if there was no change in the amount of hydroxyproline, isodesmosine or desmosine per unit volume between samples as AWUV decreased, there would still be a negative correlation with AWUV once the biochemical data were expressed per unit area. In other words, as AWUV decreases, the biochemical data per unit volume is divided by a progressively smaller AWUV value to give progressively greater biochemical amounts per unit area. Expressing the biochemical data per unit area, however, minimises the effects of variable lung inflation and is more appropriate, in physiological terms, in recognising changes within the walls themselves.

The data for collagen (both per unit volume and per unit area) were more significantly correlated with AWUV than the corresponding data for elastin. Again this may be an anatomical feature of normal lungs. Alternatively, the increase in collagen with decreasing AWUV may reflect a low level of fibrosis, associated with cigarette smoking, which is not obvious morphologically (see section 5.2).

To summarise, morphological and biochemical techniques have been used to measure airspace size, collagen and elastin on lung parenchymal tissue devoid of major bronchovascular structures. The techniques have been successful in detecting changes in connective tissue in relation to airspace size such that as the amount of alveolar tissue per unit volume decreases, there is a corresponding increase in the amount of collagen and elastin in the remaining tissue. The results may simply reflect the structural heterogeneity of the acinar unit or, in the case of collagen, may reflect a fibrotic response to injury caused by cigarette smoke.

5.2 Analysis of Collagen in Formalin Inflated Lung Samples

In order to increase the number of cases to include both non-smokers and smokers with recognisable forms of emphysema, a store of formalin inflated and fixed lungs were analysed biochemically to determine the collagen content of 40mm^3 parenchymal samples. For reasons described earlier (section 2.1.1.1) it was not possible to determine the elastin content of these samples. Unlike the agarose inflated cases, where only mean AWUV for a single inflated block of tissue was available, mean AWUV values for each of the formalin fixed lungs were determined from 6 random blocks per lobe (section 2.1.4.2).

In the non-smokers, morphometric analysis showed that there was a significant decrease in case AWUV with increasing age. These results are in agreement with Gillooly and Lamb (1993a) who established a normal range of AWUV values for non-smokers between the ages of 21 and 93 years and showed a significant negative correlation between case AWUV and age, thus demonstrating a normal increase in airspace size with advancing age in adult lungs. These authors further proposed that lungs with a case AWUV below the 95% prediction limit of the regression line should be considered as having microscopic emphysema (Gillooly and Lamb, 1993a). In another study, on a population of smokers, Gillooly and Lamb (1993b) demonstrated a similar fall in case AWUV with increasing age. Among the smokers' lungs, however, only 26% of cases had case AWUV values below the normal, age related, range for non-smokers and could, therefore, be considered to have microscopic emphysema (Gillooly and Lamb, 1993b).

In the present study, a subgroup of eight lungs had case AWUV values below the lower 95% prediction limit for non-smokers. By definition, these lungs had microscopically assessed emphysema and hence a generalised increase in airspace size. In five of these lungs there were also areas of macroscopic panacinar emphysema present, and in two of the lungs there were areas of macroscopic panacinar emphysema together with emphysematous lesions of the centriacinar type. All but one of the smokers' lungs with macroscopic centriacinar emphysema, however, had case AWUV values within the normal limits for age. According to Gillooly and Lamb (1993b), a reduction in mean AWUV in a lobe or a lung reflects a generalised increase in airspace size. Microscopically assessed emphysema, therefore, reflects a disease process which, in macroscopic form, is panacinar emphysema (where the whole of the acinar unit is affected uniformly) rather than centriacinar emphysema. Focal lesions of the centriacinar type, where respiratory bronchioles are affected, represent abnormally large airspaces in the centres of the acinar unit, with normal lung parenchyma elsewhere. Therefore, centriacinar emphysema does not in general affect overall case

AWUV (Gillooly and Lamb, 1993a). In a later study, Gillooly and Lamb (1993c) found that in a population of smokers with centriacinar emphysema, 85% had normal case AWUV values.

The subgroups analysed were (a) non-smokers, (b) smokers without macroscopic emphysema, (c) smokers with panacinar emphysema, (d) smokers with centriacinar emphysema and (e) smokers with both centriacinar and panacinar emphysema. If mean data for each group is considered then the effect of heterogeneity in lung structure and collagen content (section 5.1) is minimised. Any differences in mean Hyp content between the groups, therefore, is likely to be due to a disease process. As the interest of the present study was changes in early emphysema, samples for Hyp determination were taken from areas free from any macroscopic emphysematous lesions, on the assumption that macroscopically unaffected areas were representative of the early stages of the disease process. The four youngest of the non-smoking group were excluded from any statistical analyses in order that smoking and non-smoking groups were age matched. Thus, there were no significant differences between non-smokers and smokers as a whole, or between non-smokers and each of the smoking subgroups, with respect to age.

Samples from the smoking group had a significantly higher mean Hyp content than samples from the non-smoking group. The level of significance was greater when Hyp was expressed per unit area of alveolar wall rather than per unit volume of inflated lung. When the smoking group was sub-divided into types of emphysema, those with centriacinar emphysema and those with panacinar emphysema had significantly higher Hyp contents than non-smokers, whether Hyp was expressed per unit volume of inflated lung or per unit area of alveolar wall. Smokers with a mixture of centriacinar and panacinar emphysema had a significantly higher Hyp content than non-smokers, but only if Hyp was expressed per unit area of alveolar wall. Of the smoking group, those with panacinar emphysema had the highest Hyp content per mm^2. Although the group of smokers with no macroscopic emphysema had a higher Hyp content, both per mm^3 and per mm^2, than the non-smokers, this was not statistically significant. There was also no statistically significant difference between the smoking groups with respect to Hyp content.

A likely explanation for the increased collagen content in the lungs of smokers, particularly in the light of recent observations, is that this increase results from a fibrotic response to an inflammatory process caused by injury due to cigarette smoke.

Pulmonary fibrosis is used as a general term to describe disorders characterised histologically as having diffuse thickening of the alveolar walls. Fibrous tissue, thought to consist mostly of collagen, is localised predominantly in the interstitium (Laurent, 1986). Studies of pulmonary fibrosis in humans (Zapol *et al.*, 1979; Collins *et al.*, 1984) and in experimentally induced fibrosis in animals (Starcher *et al.*, 1978; McCullough *et al.*, 1978; Laurent *et al.*, 1981b) have shown increases in total lung collagen. Kirk *et al.* (1986) also showed increases in both total lung collagen and in collagen concentration (milligrams collagen per gram dry weight) in a large sample of patients with pulmonary fibrosis.

Indirect evidence supports an increased rate of collagen synthesis in human pulmonary fibrosis. Increased levels of collagen III propeptides have been reported in serum (Kirk *et al.*, 1984b) and lavage fluid (Low *et al.*, 1983), and increased serum levels of glucosyl transferase have also been demonstrated in patients with fibrosing lung disease (Anttinen *et al.*, 1985). In addition, Laurent and McAnulty (1983) demonstrated a decreased degradation of newly synthesised collagen in the lungs of rabbits with bleomycin induced fibrosis.

In relation to human pulmonary emphysema, evidence is emerging that fibrosis is present in alveolar tissue. Dissecting light and scanning electron microscopic observations of emphysema in humans (Nagai and Thurlbeck, 1991) demonstrated the presence of fibrotic tissue in thickened alveolar walls from 14 patients with centriacinar emphysema. Fibrous tissue was not apparent in panacinar emphysematous lesions which were present in 2 of the 14 patients. These observations call into question two aspects of emphysema. The first concerns the definition of emphysema (Snider *et al.*, 1985) which states that no obvious fibrosis accompanies destruction of alveolar walls. It is possibile that standard histological techniques are relatively insensitive for detecting increases in connective tissue proteins, unlike the biochemical analysis reported here. The second question concerns the pathogenesis of emphysema (see below).

Evidence over the last 20 years or so tends to support the protease-antiprotease hypothesis of emphysema (Janoff, 1985). If there is increased collagen, however, in some forms of emphysema at least, then perhaps a more conventional concept of inflammation may be envisaged, namely inflammatory injury followed by fibrotic healing. According to Cardoso *et al.* (1993), an increase in collagen in lung parenchymal tissue would tend to support an inflammatory repair hypothesis of emphysema whereas a decrease in elastin would tend to support the protease-antiprotease hypothesis. Cardoso *et al.* (1993) measured collagen and elastin biochemically in 147 small samples from 25 human lungs. Collagen was also

measured histochemically on an adjacent slice which was also used to determine the presence, type and severity of emphysema. Expressing the biochemical data per milligram dry weight, Cardoso *et al.* (1993) found that collagen was increased only in irregular airspace enlargement while histochemically collagen was increased in centriacinar emphysema, distal acinar emphysema and irregular airspace enlargement. Elastin (determined biochemically using desmosine and expressed as micrograms per milligram dry weight) was decreased in all grades of panacinar emphysema, in severe centriacinar emphysema, in severe distal acinar emphysema, and in irregular airspace enlargement. These authors concluded that, except in panacinar emphysema, collagen deposition and loss of elastin go together in response to injury. Furthermore, their data supported a protease-antiprotease hypothesis in panacinar emphysema, and an inflammation-repair hypothesis in centriacinar emphysema, distal acinar emphysema and in irregular air-space enlargement. These data support the idea that two different mechanisms lead to separate morphologic patterns, as suggested by Gough (1952).

Contrary to the results of Cardoso *et al.* (1993), the present study demonstrates biochemically an increase in collagen in all the types of emphysema studied (centriacinar, panacinar and mixed centriacinar/panacinar), and in smokers' lungs with no macroscopic emphysema, although the latter was not significantly different from non-smokers. These differences between the present results and those of Cardoso *et al.* (1993) may be due, in part, to the two fundamentally different ways in which the studies were carried out. The first difference relates to the mode of expression of the biochemical results. Cardoso *et al.* (1993) expressed their results per milligram dry weight of parenchymal tissue unlike the volume or area related measurements reported here. Despite Cardoso *et al.* (1993) taking the precaution of excluding samples contaminated by proteins contained in oedema, blood and inflammatory cells, it is felt that expressing the biochemical data per unit volume and per unit area of alveolar tissue gives a more informative measure of collagen within the alveolar walls compared to dry weight. The second difference relates to the population studied and the site of sampling. All samples analysed by Cardoso *et al.* (1993) were from smokers' lungs removed for cancer and comparisons were made between lesions and apparently normal areas of the same lungs. A generalised increase in collagen content would therefore have masked any increases in the collagen content of the emphysematous lungs compared to unaffected control lungs. Biochemically, increases in collagen were only observed in cases with irregular air-space enlargement, a condition that is often associated with obvious fibrosis (Snider *et al.*, 1985). In the present study, samples from emphysematous lungs were taken from areas free from macroscopic emphysema and the results compared with those from non-smokers' lungs. Interestingly, smokers with no

macroscopic emphysema had a higher collagen content than non-smokers. The increased collagen content in all types of emphysema studied here would tend to support the hypothesis of a repair process following inflammation.

The possibility that additional pathological processes lead to human emphysema, other than a protease-antiprotease imbalance, was first suggested by Snider *et al.* (1988) who used a hamster model of air-space enlargement with fibrosis induced by cadmium chloride. Previously, respiratory air-space enlargement with fibrosis had been demonstrated in humans after massive accidental exposure to cadmium (Lane and Campbell, 1954; Bulmer *et al.*, 1938). Experimental air-space enlargement with fibrosis has also been produced in guinea pigs by intratracheally instilled cadmium chloride (Thurlbeck and Foley, 1963) which has been shown to produce a lesion in rats resembling human centriacinar emphysema (Snider *et al.*, 1973). Snider *et al.* (1988) demonstrated that hamster lung elastin which had been radiolabelled with [^{3}H] valine in the neonatal period was not lost when, at a later date, air-space enlargement with fibrosis was induced by intratracheally instilled cadmium chloride. Total lung collagen and elastin were shown to be increased. Snider *et al.* (1988) concluded that enzymatically induced elastic fibre damage may not, therefore, be the cause of this form of air-space enlargement and that it is possible that the emphysema of cigarette smokers, which is predominantly of the centriacinar type, may be a form of fibrosis with air-space enlargement. Hoidal *et al.* (1985) showed that depleting hamsters of neutrophils with antineutrophil anti-serum did not inhibit air-space enlargement produced by combined cadmium chloride/β-aminopropionitrile treatment and, therefore, this type of air-space enlargement does not depend on neutrophil elastase. It has been shown that one cigarette contains, on average, 1 μg of cadmium, and of this, approximately 70% passes into smoke (Schroeder *et al.*, 1961; Nandi *et al.*, 1969). Cigarette smoke is an important source of cadmium accumulation in human organs. Compared to non-smokers, smokers accumulate more cadmium in direct relation to the number of pack-years smoked (Lewis *et al.*, 1972). For example, Hirst *et al.* (1973) demonstrated a 4-fold increase in the cadmium content of emphysematous lungs compared to controls.

Controversy surrounds the observation made by Damiano *et al.* (1986) that elastase can be immunolocalised to elastic fibres in the lungs of smokers. The observation was later confirmed by Ge *et al.* (1990), in support of the protease-antiprotease hypothesis of emphysema in smokers. In contrast, Fox *et al.* (1988) were unable to show specific binding of anti-elastase antibodies to elastic fibres in emphysematous lungs, when care was taken to block non-specific binding by using

non-immune serum that was homologous to the second antiserum. When ovalbumin was used as a blocking agent, non-specific binding to elastin of the immunogold conjugated second antibody was observed (Fox *et al.*, 1988). Damiano *et al.* (1986) and Ge *et al.* (1990) both used bovine serum albumin as the blocking agent and, therefore, their results may be due to non-specific binding.

It remains the case that most research effort has been, and continues to be, based on the hypothesis that a protease-antiprotease imbalance is responsible for emphysema in smokers. The evidence for this hypothesis appears to be so compelling that many investigators have explored ways to prevent emphysema by manipulating the protease-antiprotease balance (Snider *et al.*, 1990). The main approach that has been considered is that of supplementing the antiprotease system of the lungs, and to this end a synthetic antiprotease is already in the early phase of clinical trials (Snider, 1992).

The present study and those of Nagai and Thurlbeck (1991) and Cardoso *et al.* (1993) support the idea that following inflammatory injury there is some attempt at repair. An inflammatory-repair hypothesis does not necessarily contradict the protease-antiprotease hypothesis and it seems likely that the morphological and biochemical features of emphysema in smokers can be explained by a combination of both hypotheses. Thus, destruction of extracellular matrix components, in particular elastic fibres, by excess protease activity leads to loss of alveolar architecture and an increase in air-space size. Fibrotic healing, characterised by an increase in alveolar collagen content and possible repair of elastic fibres, cannot however restore normal alveolar architecture and may even exacerbate alveolar loss by distortion of normal areas rendering them more prone to attack by elastases.

All samples in the present study were taken from areas that were free from macroscopic emphysema, regardless of whether emphysematous lesions were present elsewhere in the lungs. Therefore, the increases in collagen in these areas of smokers' lungs compared to non-smokers seems to suggest that an inflammatory-fibrotic repair process occurs in the early stages of emphysema before proteolysis of elastic fibres.

In summary, the present study has demonstrated that parenchymal samples taken from areas of smokers' lungs without macroscopic emphysema have a significantly higher collagen content than samples from non-smokers' lungs. Samples taken from smokers' lungs which also have areas of macroscopic emphysema had the highest collagen content. The higher collagen content may represent fibrotic tissue in thickened alveolar walls as a result of inflammatory injury caused by cigarette smoke

followed by fibrotic healing. The fact that an increase in collagen content was demonstrated in areas without macroscopic emphysema suggests that a possible inflammatory repair process precedes elastic fibre destruction and/or damage ultimately leading to macroscopic emphysema.

5.3 Analysis of α1-Protease Inhibitor (α1-Pi) Deficient Lungs

Lungs from three subjects with α1-Pi deficiency, and lungs from two smokers and one non-smoker, were analysed biochemically and morphometrically for the determination of Hyp, isodesmosine, desmosine and AWUV. For the determination of Hyp, isodesmosine and desmosine, lungs were inflated with 70% (v/v) ethanol. Due to technical difficulties in inflating lung tissue with ethanol, especially lung tissue from α1-Pi deficient subjects, AWUV measurements were not possible on these lobes. Therefore AWUV and further Hyp determinations were made on second lobes inflated in the standard manner with formalin (section 2.1.4.2).

Despite concerns about possible variable inflation of the ethanol inflated lobes (M.R. Lang, personal communication), there was no statistically significant difference between Hyp content per mm^3 of ethanol inflated lobes compared to corrresponding formalin inflated lobes. It was felt, therefore, that the isodesmosine results from the ethanol inflated lobes accurately reflected the isodesmosine contents of the corresponding formalin-treated lobes.

The α1-Pi group as a whole had a significantly lower AWUV and a significantly higher Hyp content (per mm^3 and per mm^2) than both the non-smoker and the smokers as a group. Compared to the non-smoker, however, the α1-Pi group did not have a significantly different isodesmosine content (per mm^3 and per mm^2). Compared to the non-smoker and the α1-Pi group, the smokers as a group had a significantly higher isodesmosine content (per mm^3 and per mm^2).

It should be noted that because the genotypes and smoking histories of the α1-Pi deficient subjects have not yet been established, it cannot be stated whether emphysema was solely a result of α1-Pi deficiency and/or a result of smoking. The extent and severity of emphysema compared to the smokers (section 4.4) would tend to suggest that α1-Pi deficiency would at least exacerbate emphysema caused by cigarette smoking. The increase in collagen content compared to the non-smoker is consistent with the presence of an inflammatory-repair process (section 5.2). As only one non-smoker was included in this part of the study, the results must be regarded as preliminary. This is the first study to demonstrate, however, no change in the elastin

content of emphysematous lungs with α1-Pi deficiency, compared to non-smoking, unaffected controls.

Chrzanowski *et al.* (1980) and Tarjan *et al.* (1989) found that the elastin content of lung parenchymal tissue from subjects with panacinar emphysema, including subjects with α1-Pi deficiency, was decreased from normal when expressed as a proportion of crude connective tissue. In view of the present findings it seems likely that the simple explanation for these authors' findings is that the decrease in the proportion of elastin in the crude connective tissue samples was due to an increase in the collagen content of the samples rather than a decrease in the elastin content. These authors did not measure the collagen content of their samples. Of course it cannot be stated that destruction of lung elastin does not take place.

A possible explanation for the present findings is that the processes leading to the presumed destruction of lung elastin are being counterbalanced by synthesis of elastin in a manner that does not restore alveolar architecture. Alternatively, there is a rearrangement of existing elastin as alveolar walls undergo possible structural remodelling caused by damage to, rather than destruction of, elastic fibres (Fukuda *et al.*, 1989).

One question which still remains to be answered is: Why do only 15% of long term smokers develop chronic obstructive pulmonary disease (COPD) including emphysema (Snider *et al.*, 1990)? Also, of the total population with severe α1-Pi deficiency, only a few per cent present medically for COPD (Eriksson, 1965; Mittman *et al.*, 1974). Although PiMZ heterozygotes have intermediate serum levels of α1-Pi, current evidence indicates that this is not associated with an increased risk of developing COPD (Bruce *et al.*, 1984). It may be that healthy PiMZ heterozygotes do not expose themselves to lung injury by smoking and therefore, intermediate levels of α1-Pi are sufficient to protect the lung against proteolytic attack.

The susceptibility of certain smokers to emphysema may have a genetic basis. Two independent studies have recently shown that certain restriction fragment length polymorphisms in the α1-Pi gene occur more commonly in patients with COPD even though they express the common MM phenotype and have normal serum levels of α1-Pi (Poller *et al.*, 1990; Kalsheker *et al.*, 1990). The 15% of smokers who have COPD may therefore represent a group with a genetic predisposition to COPD which is triggered by long term smoking.

The collagen content of lung parenchymal tissue from subjects with α1-Pi deficiency was found to be significantly increased compared to that of the non-smoker.

This increase may, like the increase seen in the smokers of section 4.4, reflect the presence of fibrotic tissue as a result of a repair process following inflammation. One important difference between these smokers and the α1-Pi deficient subjects, however, was that the samples from the smokers' lungs were taken from areas free from macroscopic emphysema, unlike the α1-Pi deficient lungs where all areas were affected. Therefore results on the smokers' lungs may be more indicative of the early disease processes, while results from the α1-Pi deficient lungs may be more indicative of end stage disease.

In summary, preliminary data suggest that elastin may not necessarily be lost from the lung parenchyma of subjects with emphysema associated with α1-Pi deficiency. One possible explanation for this finding may be that loss of elastin is counterbalanced by resynthesis without restoration of normal alveolar architecture. A second possible explanation is that there is a rearrangement of existing elastin as alveolar walls undergo structural remodelling caused by damage to, rather than destruction of, elastic fibres.

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APPENDIX

Published Papers

M.R. Lang, G.W. Fiaux, D.J.S. Hulmes, D. Lamb and A. Miller (1993). Quantitative studies of human airspace wall in relation to collagen and elastin content. *Matrix* **13:** 471-480.

M.R. Lang, G.W. Fiaux, M. Gillooly, J.A. Stewart, D.J.S. Hulmes and D. Lamb (1994). Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs. *Thorax* **49:** 319-326.

Abstracts

M.R. Lang, G.W. Fiaux, D.J.S. Hulmes and D. Lamb (1992). A study of the lung extracellular matrix in relation to tissue density (Abstract). *American Review of Respiratory Disease* **145** (Suppl): A762.

M.R. Lang, G.W. Fiaux, D. Lamb and D.J.S. Hulmes (1993). Lung collagen in non-smokers and smokers with emphysema (Abstract). *Thorax* **48:** 418.

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M.R. Lang, G.W. Fiaux, J.A. Stewart, M. Gillooly, D. Lamb and D.J.S. Hulmes (1993). Is fibrous tissue increased in emphysematous lungs? (Abstract). *Journal of Pathology* **170** (Suppl): 277.

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Quantitative Studies of Human Lung Airspace Wall in Relation to Collagen and Elastin Content

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Abstract

Biochemical determinations of the collagen and elastin content in 50 mm^3 samples of human lung are presented in relation to morphometric measurements of lung structure, as the amount of alveolar wall surface area per unit volume (AWUV), on adjacent slices. There were no differences in AWUV values, collagen content (determined as hydroxyproline) or elastin content (determined as isodesmosine) between upper and lower lobes within a single lung. In a study of 102 samples from 9 smokers lungs with no evidence of macro- or microscopic emphysema (as estimated by AWUV measurement), there was a negative correlation between AWUV and the amounts of collagen or elastin per unit volume of inflated lung. The correlation was stronger when collagen and elastin content were expressed per unit area of alveolar wall. The negative correlation is interpreted as representing either the anatomical variation within the complex hierarchy of normal lung structure or possibly low levels of fibrosis in response to cigarette smoking.

Key words: collagen, elastin, lung, morphometry.

ntroduction

Collagens and elastin are the major extracellular matrix CM) proteins of lung (Zapol et al., 1979; Kirk et al., 36; John and Thomas, 1972; Pierce et al., 1961; Pierce Ebert, 1965). These proteins play vital physiological s in lung mechanics, enabling the tissue to stretch during ation, preventing over-inflation, and facilitating subse- nt recoil (Snider and Karlinsky, 1977). Various studies e been directed towards identifying changes in the ounts of collagen and elastin, during ageing and pulmo- y disease. Few studies, however, have substantiated any nge from normality at a biochemical level, with the ssment of lung structure being rather subjective and quantitative (Saetta et al., 1985; Saito et al., 1989; gai et al., 1989). The purpose of the present study is to te morphometric techniques for quantifying alveolar cture at the microscopic level to corresponding hemical measurements of collagen and elastin content.

Many changes have been observed, both morphologically and functionally, in the ageing human lung. One major change is the progressive loss in the proportion of tissue occupied by the alveolar walls. It has been estimated morphometrically by Thurlbeck (1967b) that between the ages of 20 and 80 one third of total airspace wall surface area is lost. Gillooly and Lamb (1993a) also found that airspace wall per unit volume decreased by one third between the ages of 20 and 90. This loss of alveolar walls, with increase in airspace size, results in a reduction of surface area available for gas exchange. A loss of the lung's elastic recoil (at 60% total lung capacity) is also age dependent (Turner et al., 1968; Zapletal et al., 1971). It is likely that the loss of both airspace wall and elasticity must in some way reflect alterations in the structure and composition of the ECM.

The diseased state pulmonary emphysema, defined in pathological terms as

> "a condition of the lung characterised by abnormal enlargement of the airspaces distal to the terminal respiratory bronchiole accompanied by destruction of the walls, without obvious fibrosis" (Snider et al., 1985),

is usually induced by cigarette smoking. It has been shown that this disease process further accelerates the loss of airspace wall with age (Gillooly and Lamb, 1993 b). Biochemical comparisons, however, of collagen and elastin content between non-emphysematous and emphysematous lungs have usually failed to identify any changes in these ECM components, in spite of abnormalities in microscopic structural appearance. An early study on whole lung homogenates (Pierce et al., 1961) found no changes in total collagen and elastin levels in emphysematous and non-emphysematous lungs. More recently Cardoso et al. (1993) assessed lungs microscopically for emphysema, and made quantitative biochemical measurements of collagen and elastin within lesions from 4 types of emphysematous lungs. They reported an increase in collagen with irregular emphysema (IE) and a reduction in elastin with both IE and centriacinar emphysema (CAE), per dry weight of tissue. These authors also observed that although fibrosis was obvious microscopically it was not detected biochemically in lesions other than IE.

Any attempt to account for both age and disease related changes in relation to biochemical alterations in ECM components must take account of the complex hierarchy of lung structure (Weibel, 1959). Histological assessment of emphysematous lung has usually only been qualitative, even though quantitative morphometric techniques have been available for some time. Collagens and elastin are distributed non-uniformly throughout the bronchial tree (Laurent, 1986; Starcher, 1986) therefore, in order to identify any morphological and biochemical changes within the airspace walls, it is important to select only parenchymal tissue that is free of pleura and major bronchovascu structures. Problems associated with inappropriate sar ling of the lung may account for some of the conflicting d in the literature on the amounts of ECM components ageing and disease. Figure 1 shows the microanatomy of lung acinar unit, depicting the successive change in str ture from proximal to distal regions of the unit.

The choice of denominator is of crucial importance in quantitation of lung ECM components. In emphysema, example, changes in collagens and elastin are difficult interpret when expressed per weight of lung tissue. A m useful denominator would be either inflated lung volume some quantitative measure of the amount of airspace w We have recently developed a fast interval processing te nique for the quantitation of airspace wall surface a (Gillooly et al., 1991). Here we describe procedures for biochemical analysis of collagens and elastin and th expression in relation to a quantitative measure of airsp wall, AWUV (Airspace Wall surface area per Unit Volu of lung tissue).

Materials and Methods

Sources of tissue

Lungs or lobes were obtained from 9 patients who h undergone surgery for the removal of small periphe tumours. All patients were smokers within the range 55–85 and showed no evidence of macrosco emphysema. An additional two lungs were obtained autopsy from a 75-year old smoker and an 85-year old n smoker.

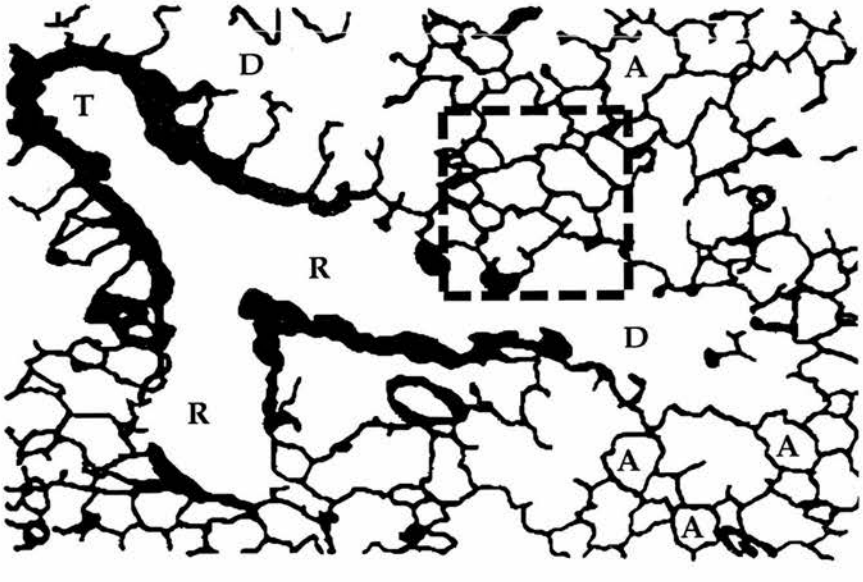
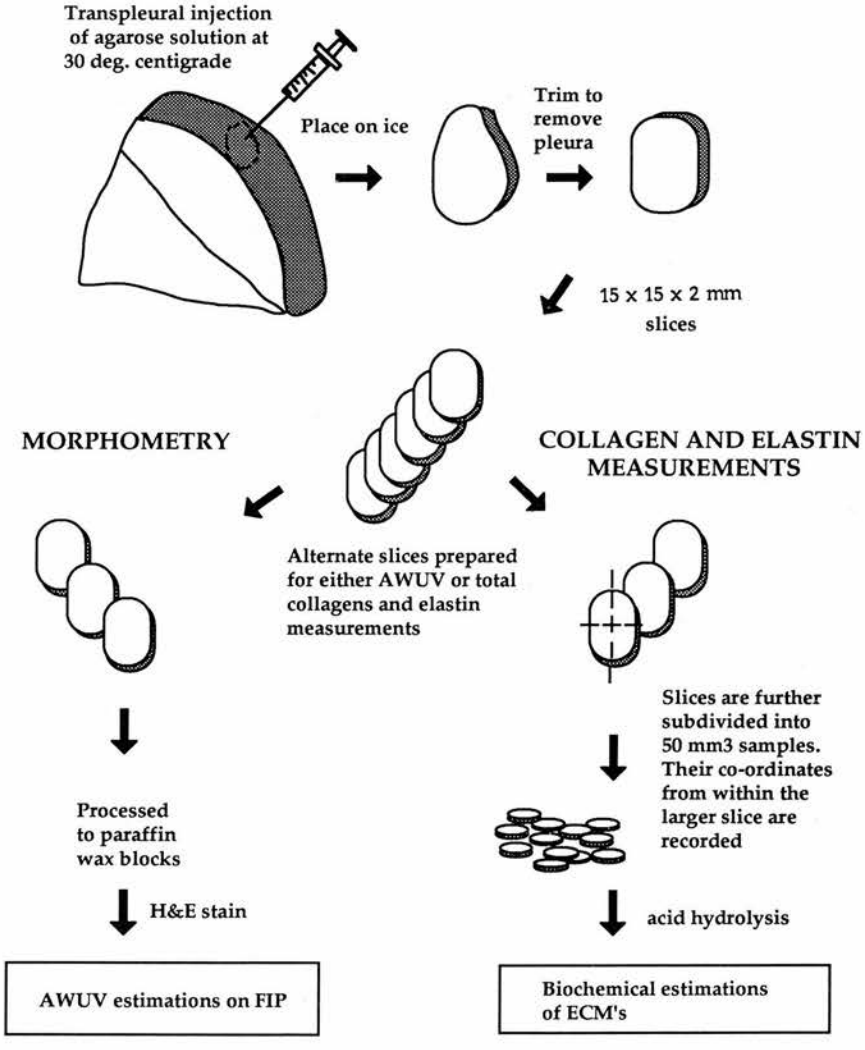


Fig. 1. Digital binary image from a tological section (H & E stain) of hu lung parenchyma showing the succes change in structure from proximal to d end of the acinar unit. The frame (da box) represents a typical 1 mm^2 fiel tissue. Mag. x40.
KEY
T: terminal bronchiole, R: respira bronchiole, D: alveolar duct, A: alveol

Lung preparation and sampling

In order to express the biochemical data in terms of lung ucture it was necessary to develop a technique whereby easurements of total collagen and elastin could be carried t on known volumes of fresh, inflated lung tissue. The ndard procedure of inflation (Wentworth, 1950) by rabronchial perfusion with buffered neutral formalin NF), was unsuitable in this study in view of the reaction formaldehyde with the amino groups of lysine (Kadler d Chapman, 1988) and hence possible interference with termination of the elastin specific cross-links desmosine d isodesmosine. A technique was required for the prepa-ion of fresh lobes or portions of lobes in a condition table for both morphometric and biochemical analysis. e method developed is described in detail below.

Following resection, lung tissue was immediately placed on ice and on arrival at the laboratory was transferred to a high risk fume cabinet. A random zone of the lung (approximately 100 × 50 × 50 mm) was then transpleurally inflated away from the tumour with an injection of 2% agarose solution (approximately 150–200 ml depending upon specimen size) previously heated to 30 °C. The zone was considered inflated when the pleural surface became firm and smooth in appearance. The tissue was maintained on ice for a further 10 min until the agarose had solidified within the airspaces, effectively maintaining inflation. The inflated zone was then dissected free from the surrounding lung, trimmed of pleura, and stored at 4 °C for a further 20 min. After placing the zone on a purpose built template, slices approximately 15 × 15 × 2 mm were taken with a



2. Schematic representation of the hod of sample preparation of fresh lung ıe for morphometric measurement of UV and biochemical estimations of ıgen (hydroxyproline) and elastin lesmosine).

surgical skin graft knife blade (Downs Surgical, Mitcham, England). The position of each slice within the zone was recorded and alternate slices were stored in either 10% BNF (for histology and subsequent morphometry), or 70% ethanol (for collagen and elastin determinations). In order to study lung composition within smaller defined regions, the tissue prepared for biochemical measurements was further subdivided into approximately 50 mm^3 samples (5 × 5 × 2 mm) with a surgical blade. The exact dimensions of these subdivisions were determined with callipers and their position within the larger slice recorded. At this stage the morphology of a histological section from the adjacent slice was examined microscopically and those regions containing obvious, large amounts of bronchovascular structures were excluded. From 9 lungs, 102 samples were prepared by the above method. Figure 2 shows a schematic representation of the sample preparation procedure.

Sampling of post mortem lungs

Whole lungs were obtained at autopsy in order to study any anatomical variations in AWUV, collagen or elastin. Three zones (upper, middle and lower) within each of the upper and lower lobes were inflated with 2% agarose solution. The zones were placed on ice and processed to 2 mm thick slices as described previously. Alternate slices (15 × 15 × 2 mm) were taken for either morphometry or biochemical estimations. Slices for collagen and elastin determinations were further subdivided into 50 mm^3 samples. Upper and lower lobes of each lung were compared by one-way analysis of variance (ANOVA). The mean AWUV of individual subdivisions (5 × 5 mm) within a slice (15 × 15 mm) comprised at least 20 AWUV estimations (see below).

Preparation of tissue for morphometry

Formalin fixed slices of agarose inflated lung were dehydrated through a graded ethanol series and embedded in paraffin. From each slice histological sections were cut at 4 μm on a rotary microtome. The sections were de-waxed in xylene and stained by the haematoxylin and eosin method (Culling, 1963). AWUV values were determined for each 1 mm × 1 mm field in an 11 × 11 mm region within each section. Tissue shrinkage occurs during processing into paraffin wax. Therefore, it was necessary to calculate a shrinkage factor for each section and adjust the morphometric data accordingly. Tissue slice dimensions were measured before and after processing, and used to calculate the area shrinkage factor and, its square root, the linear shrinkage factor. AWUV values were multiplied by the reciprocal of this linear factor (Gillooly et al., 1991).

AWUV measurement by Fast Interval Processor (FIP)

AWUV values were measured by the Fast Interval Proce sor (FIP; Tucker and Shippey, 1981), a prototype version a commercially available fully automated rapid scanni device, the Cytoscan (Image Recognition Systems, Wa rington, Cheshire, England). The system was specifica modified to enable lung alveoli to be quantified on histolo ical sections. It consists of a MIPROC microprocess (Plessey) linked to a Nikon inverted microscope with motorised stage and a Fairchild CCD linear image senso Photosensitive units within the sensor recognise optic density pattern in the specimen creating a digitised ima consisting of picture elements or 'pixels'. A user-defin threshold limit determines what is recognised as bac ground or tissue. A size filter then reduces 'noise' eliminating particles (such as debris and lung inflammato cells) less than 6 μm in diameter thus ensuring that on intercepts with tissue are counted by the electronic test lin

The FIP uses the method of mean linear intercept (Li Aherne and Dunnill, 1982), whereby intercepts of tiss with a test line are counted, and the average distan between intercepts is determined. Tissue surface area p unit volume is then derived from Lm. A more detail description of the FIP is given by Gillooly et al. (199 Perimeter values for each 1 mm^2 field were stored on t FIP, transferred to a mainframe computer (Unix™) a AWUV values were calculated for each 1 mm^2 field (such the 1 mm^2 region of alveolar tissue outlined in Fig. 1). 1 AWUV values for each section were displayed and me AWUV values were calculated for each 5 × 5 mm subdi sion, for comparison with biochemical data from cor sponding sub-divisions of adjacent lung slices. Values Lm and AWUV were derived as follows:

Lm = Total test-line length / Total number of intercep

Surface area (SA mm^2) = 2 V / Lm, where V = volu (mm^3).

Airspace **W**all surface area per **U**nit **V**olume (AWUV) 2/Lm (mm^2/mm^3).

Determinations of total collagens

Total collagen was measured using 4-hydroxyprol (hyp) as a relatively specific marker. Hyp values w obtained either by complete amino acid analysis or selective determination of secondary amino acids.

Amino acid analysis was performed on a model 42 amino acid analyser (Applied Biosystems, Warringto Briefly, the samples were de-fatted and homogenised acetone. A known amount of nor-leucine (BDH Ltd., Po England) was added as an internal standard and the sa ples were hydrolysed in 6 N HCl (110 °C for 24 h) in vapour phase. The samples were reconstituted in 20 0.025% K_3EDTA, passed through a 0.22 μm filter (Geln Sciences Ltd., Northampton, England), diluted a 100-f

stilled water and 5 µl was applied to the analyser. The
no acids were labelled automatically with phenylisocyanate (PITC) and the resulting phenylthiocarbamyl-
no acid derivatives (PTC-AA) were separated on a
nm (I.D.) × 22 cm cartridge-style reverse-phase col- packed with C18 bonded phase silica. The peaks were
lved by gradient elution (I. Davidson, personal com-
ication) as follows: 0–4 min, 2% buffer B (70% v/v
onitrile in 32 mM sodium acetate, pH 6.1); 4–10 min,
buffer B; 10–20 min, 35% buffer B; 20–25 min,
buffer B; 25–30 min; 100% buffer B. Buffer A con-
d of 3% (v/v) acetonitrile in 50 mM sodium acetate,
.22. The eluate was monitored at 254 nm. The amount
ach amino acid was determined from peak areas in
ion to known standards and corrected for recovery of
nor-leucine internal standard.
pproximately 50% of the hyp data were obtained on
mino acid analyser. The remainder were obtained by
tive determination of secondary amino acids using 9-
roenylmethylchloroformate (FMOC-Cl) derivatisation
rlink et al., 1989). In this procedure a known amount
,4-dehydroproline (DHP; Sigma Chemical Co. Ltd.,
e, England) was added to the samples as internal stan-
. Samples were hydrolysed in liquid phase 6N hydro-
ric acid for 24 h at 110 °C, dried in a Speed Vac Con-
rator (Savant Instruments Inc., Farmingdale, NY, USA)
reconstituted in 200 µl 0.025% K_3EDTA. The deriva-
ion procedure and chromatography conditions were a
ification of the method of Teerlink et al. (1989). HPLC
ysis was performed on a 4.6 mm ID × 25 cm Dynamax-
A column (Rainin Instrument Co. Inc., Woburn, MA,
) packed with 5 µm C18 bonded spherical silica using a
on model 306 solvent delivery system and a model 231
sampler (Gilson Medical Electronics S.A., Villiers-le-
France). The peaks were resolved isocratically at a flow
of 1.0 ml/min, using 30% (v/v) acetonitrile in 50 mM
c acid (adjusted to pH 4.3 with 1 M NaOH). Fluores-
e of the eluate was monitored with an LC 240 fluores-
e detector (Perkin-Elmer Ltd., Maidstone, Kent, Eng-
) using an excitation wavelength of 260 nm and an
sion wavelength of 330 nm. Results were calculated
calibration curves, constructed with known amounts
yp versus the ratio of the peak areas of hyp and a fixed
unt of 3,4-DHP.

etermination of elastin

esmosine and isodesmosine, determined as elastin
fic marker cross-links, were also determined by HPLC.
ples were hydrolysed as above (liquid phase hydrolysis)
treated by the cellulose "mini-column" method of
ner (1982) to remove interfering substances.
nosine and isodesmosine were analysed by HPLC using
dification of the method of Yamaguchi et al. (1987)
the same column and solvent delivery system as for hyp determination following FMOC derivatisation. The peaks were resolved isocratically, at a flow rate of 0.8 ml/min, using 18% (v/v) acetonitrile / 82% (v/v) 0.1 M sodium phosphate containing 20 mM SDS, final pH 4.5. The eluate was monitored at 270 nm with a model 759A u.v. absorbance detector (Applied Biosystems Inc., Foster City, CA, USA). Results were calculated from calibration curves, constructed with known amounts of pure desmosine and isodesmosine (Elastin Products, Owensville, MO, USA) versus peak area.

Presentation and expression of data

Biochemical measurements of alveolar collagen and elastin were expressed per mm^3 of agarose inflated tissue. Protein amounts per unit area of airspace wall (amino acids/mm^2) were obtained by dividing the amino acids/mm^3 values by corresponding AWUV values (mm^2/mm^3).

Statistics were carried out on Minitab™ and StatWorks™ packages.

Results

Assessment of the techniques

The inter-observer reproducibility of AWUV determined by FIP was assessed by repeating the scans on a series of 100 random sections from 11 lungs with two different operators. The intra-observer reproducibility was estimated by scanning the same section 20 times by the same operator.

The FIP AWUV data from the two observers were compared and found to be highly linearly related ($r = 0.986$, $p < 0.001$) and were within 1% of each other. When the data

Table I. Intra- and inter-assay variation in collagen and elastin determinations on independent hydrolysates of alveolar tissue.

	PTC-hyp[a]	FMOC-hyp[b]	isodesmosine
(a) Intra-assay			
CV[c]	13.85	6.3	9.0
n[d]	17	11	76
(b) Inter assay			
CV	9.3	4.3	9.7
n	7	28	14

Amounts of hydroxyproline (hyp) and isodesmosine were determined on approximately 5 × 5 × 2 mm sub-divided slices of agarose-inflated lung. The PTC-hyp and FMOC determinations were made on independent samples. Intra-assay and inter-assay variations were assessed partly by the method of Percy-Robb et al. (1980).

[a] PTC-hyp = phenylthiocarbamyl-hyp
[b] FMOC-hyp = 9-fluoroenylmethylchloroformate-hyp
[c] CV = coefficient of variation
[d] n = number of assays

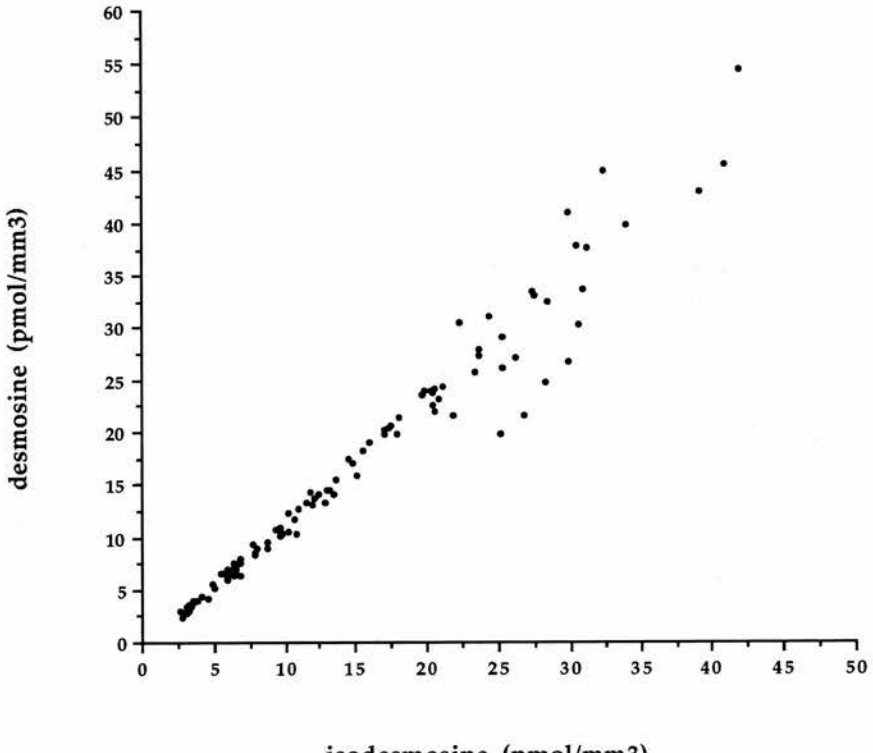


Fig. 3. Relationship between the two e
tin markers, desmosine and isodesmo
within 102 alveolar samples from 9 lun

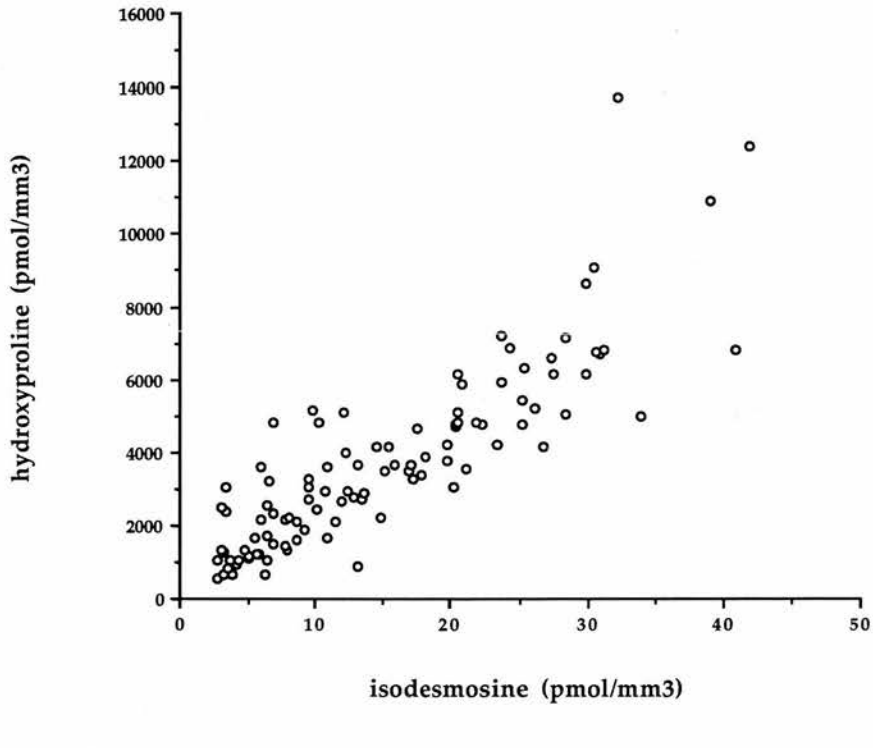
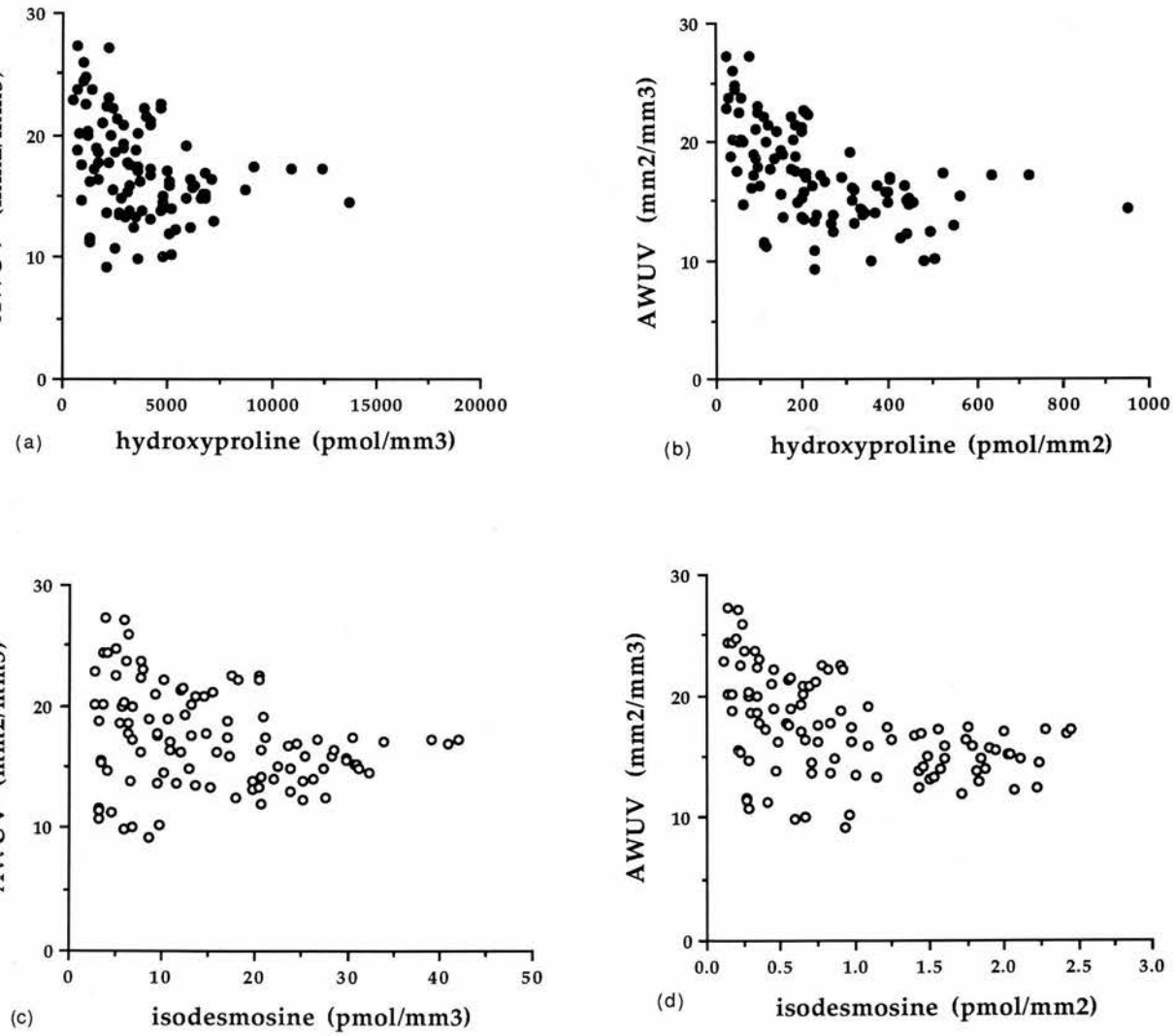


Fig. 4. Correlation between alveolar c
gen (hydroxyproline) and elastin (isc
mosine) content for 102 samples f
9 lungs.

e II. Anatomical variation in AWUV, alveolar collagen (hydroxyproline) and elastin (isodesmosine) content within individual lungs a 75 year old smoker and an 85 year old non-smoker.

	75-year old smoker			85-year old non-smoker		
	AWUV mm^2/mm^3	hyp (pmol/mm^3)	ide (pmol/mm^3)	AWUV (mm^2/mm^3)	hyp (pmol/mm^3)	ide (pmol/mm^3)
	18.61 (1.85)	7080 (2500)	27.7 (4.8)	14.74 (1.04)	6440 (640)	25.9 (6.7)
	17.86 (2.64)	7150 (3450)	30.2 (10.7)	15.23 (1.58)	5876 (1270)	28.0 (4.2)

reviations:
= upper lobe, LL = lower lobe
UV = amount of alveolar wall per unit area
= hydroxyproline
= isodesmosine
represents means with standard deviation
12 protein estimations from each lobe.



. AWUV as a function of hydroxyproline and isodesmosine, in cumulative data from the 9 resected cases, 102 samples, (a) hydroxy-ne per mm^3 (b) hydroxyproline per mm^2 (c) isodesmosine per mm^3 and (d) isodesmosine per mm^2.

were compared by Mann-Whitney U Test there was no significant difference between the two observers ($p = 0.68$). Variation in AWUV data from the sections scanned 20 times by the same observer was 0.72%.

Since some of the earlier hydroxyproline data (approximately 50% of the total) were obtained on the amino acid analyser (rather than selective determination of secondary amino acids), results obtained by each method on identical samples were compared. When hydroxyproline data from 10 samples were compared in this way, Mann-Whitney U Test showed that the results obtained by either method did not differ significantly ($p = 0.97$) and therefore hydroxyproline results from both methods were combined. Table I shows the intra-assay and inter-assay variations in the PTC-hyp, FMOC-hyp and isodesmosine determinations.

Regional variation within a lung

Table II shows the AWUV values, collagen and elastin content for upper and lower lobes of two *post mortem* lungs. There were no significant anatomical differences in AWUV ($p = 0.50$), collagen per mm^3 ($p = 0.95$), or elastin per mm^3 ($p = 0.40$) between the upper or lower lobes of the 75-year old smoker, or within the lung of the 85-year old non-smoker ($p = 0.55$, $p = 0.27$ and $p = 0.37$, respectively).

Analysis of several cases

The collagen and elastin contents for 102 samples from 9 resected lungs are shown below. Data for the two elastin markers, desmosine and isodesmosine, are closely correlated (Fig. 3; $r = 0.979$, $p < 0.001$) and in an approximate 1:1 ratio. In the results below, total elastin is represented by isodesmosine (ide). The relationship between total collagens and elastin is shown in Fig. 4. The data show a strong relationship between estimations of the two proteins and demonstrates their degree of co-distribution within alveolar tissue at the level of the 50 mm^3 sampling size.

Cumulative data for AWUV in relation to both collagen and elastin content are shown in Fig. 5. The data for collagen and elastin are expressed in two ways. First, as shown in Fig. 5 (a), total hyp within each 50 mm^3 sample is expressed per unit volume (mm^3) of lung tissue. Second, as shown in Fig. 5 (b), hyp amounts per unit volume have been divided by AWUV to give hyp per unit area of alveolar wall (mm^2). When hyp data were expressed per unit volume, $r = -0.326$ ($p < 0.001$) and when expressed per unit area, $r = -0.530$ ($p < 0.001$). Fig. 5 (c) and (d) show corresponding data for elastin, with $r = -0.265$ ($p < 0.01$) per unit volume and $r = -0.459$ ($p < 0.001$) per unit area. The data show that when collagen and elastin are expressed either per unit volume or per unit area of airspace wall, there is a negative correlation between changes in AWUV and the amounts of these ECM proteins, i.e. as AWUV drops over the measured range there is a corresponding increas alveolar collagen and elastin content. The significanc the negative correlation is greater when protein amo are expressed per unit area of airspace wall.

Discussion

We have determined the biochemical amounts of c gen and elastin in human lung in relation to the amou alveolar wall per unit volume (AWUV). The work is pa a larger study of the changes in extracellular matrix (E in relation to morphological changes in human lung space wall, particularly in emphysema. As the amou alveolar wall falls in emphysema (Thurlbeck, 1967 a, Gillooly and Lamb, 1993 a), and as the lung loses elasti it would be reasonable to expect a similar fall in E content. The pathogenic mechanisms proposed for emphysematous process suggest that the elasticity of lung is lost as a consequence of the release of neutro and/or macrophage proteolytic enzymes into the extra lular matrix (Wright, 1961; Hayes et al., 1975; Kuhn e 1976; Soskel and Sandberg, 1987), though there is quantitative biochemical data about changes in ECM teins to substantiate this theory. In order to investigate changes, it was first necessary to establish the rela between ECM content and AWUV in lungs without em sema. The tissue was prepared by agarose inflatio enable both collagen and elastin to be expressed per volume or per unit surface area of inflated lung u previous biochemical determinations where data were malised to tissue dry weight (Cardoso et al., 1993) or w lungs (Pierce et al., 1961). In addition, care was take ensure that the samples used in the analysis were devoi the major bronchovascular support structures of the l which are rich in ECM.

There were no significant variations in AWUV or amounts of collagen and elastin per unit volume of when data averaged from several slices in both upper lower lobes were compared (Table II). We conclude f these findings that in man gravitational stress on the has little influence on the distribution of alveolar coll and elastin. These observations are consistent with tho Kirk et al. (1986), who also showed no difference in c gen either between lobes or vertically throughout the l Within slices however, significant variations in AV were detected at the level of the $5 \times 5 \times 2$ mm sub sions, though average AWUV values for whole slices (15×2 mm) were relatively constant (Lang and L unpublished observations). In view of this local variati AWUV, collagen and elastin data for the 5×5 mm sub sions were related to AWUV data for corresponding 5 mm fields in the adjacent slice.

Analysis of samples from 9 resected lungs show negative correlation between sample AWUV and

ounts of collagen (measured as hydroxyproline) or elas- (measured as isodesmosine), whether expressed per unit ume or per unit surface area of alveolar tissue. Collagen l elastin were found to co-distribute, though the negative relation with AWUV was more significant for the colla- data. Samples with low AWUV contained more colla- and elastin (per unit volume of lung or per unit area of eolar wall) than samples with high AWUV.

We propose two possible interpretations of these find- s. First, within normal lung, areas of high AWUV occur listal regions of the acinar unit and these may be less rich ECM proteins (per unit volume) than the more proximal ions (containing vascular material and supporting struc- es such as respiratory bronchioles) with low AWUV. e resected lung samples showed no obvious signs of croscopic emphysema, and the AWUV values for the rose inflated zones were within the normal range for n-smokers (Gillooly and Lamb, 1993a). Therefore our ervations may simply reflect the complex hierarchical ucture of the acinar unit. Second, the biochemical data y be detecting a low level of fibrosis in the low AWUV mples that may not be obvious morphologically, as indi- ed by the increased amount of ECM per unit volume of ated lung.

The negative correlation with AWUV is more significant en protein amounts were expressed per unit area of eolar wall. It should be noted that even if there is no relation between AWUV and ECM per unit volume, n a negative correlation with AWUV may appear when M per unit volume is divided by AWUV to give ECM per t area of alveolar wall. Samples with constant amounts ECM per unit volume will have greater amounts of ECM unit area as the AWUV value decreases. However, pressing ECM amounts per unit area of alveolar wall has advantage of minimising the effects of variable lung ation, and is more physiologically appropriate in recog- ng changes within the walls themselves. We feel that the plication of these techniques will facilitate the under- nding of changes in lung structure in relation to collagen l elastin in patients with early to late stage emphysema.

Acknowledgements

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Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs

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Abstract

Background **– Emphysema is currently defined as "a condition of the lung characterised by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, and without obvious fibrosis." The functional and morphological changes that occur in emphysema have largely been attributed to changes in alveolar elastin rather than in collagen. A study was performed to determine whether the amount of collagen in the alveolar wall changes with age in the lungs of non-smokers and of smokers with different types of macroscopically defined emphysema in relation to a microscopic measurement of lung structure.**

Methods **– Total alveolar wall collagen was measured (as hydroxyproline) in known volumes of distended lung tissue (by reverse phase high pressure liquid chromatography) in the lungs of non-smokers (n = 23) and in regions sampled away from emphysematous lesions in the lungs of 36 smokers (four with no emphysema, 13 with centriacinar emphysema (CAE), nine with panacinar emphysema (PAE), and 10 with a mixture (MIX) of both PAE and CAE). Mean lung airspace wall surface area per unit volume (AWUV) was calculated from at least six random blocks per lung and on histological sections immediately adjacent to those prepared for collagen measurement with a rapid scanning device (fast interval processor).**

Results **– In non-smokers there was no significant correlation between the amount of collagen in the alveolar wall tissue and either mean lung AWUV or increasing patient age when amounts of collagen were expressed either per unit volume of distended lung (40 mm³ sample) or per unit surface area of airspace wall tissue. Smokers without emphysema had similar amounts of collagen to non-smokers. Lungs with PAE and MIX, but not CAE alone, contained significantly more collagen than normal when expressed per unit volume of airspace wall tissue whereas all groups, including CAE, contained significantly raised amounts of collagen when expressed per unit surface area.**

Conclusions **– There is no significant age related change in the collagen content of the lungs of non-smokers which suggests that, as AWUV is lost with age, the main collagenous framework is maintained. However, in smokers with emphysema there is a loss of airspace wall tissue in regions remote from the macroscopic lesions that is accompanied by a net increase in collagen mass. The greater accumulation of collagen in MIX lungs than in CAE lungs suggests a greater degree of structural damage, indicative of an alternative pathogenetic mechanism operating between the different types of emphysema. Our results suggest an active alveolar wall fibrosis in emphysema as a consequence of cigarette smoking. It is suggested that the definition of emphysema may require further revision to include such change.**

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The presence of collagen in the extracellular matrix is fundamental to the normal structural integrity, compartmentation, and functional capacity of the lung. Collagens are found in abundance in airways, vessels, pleura, basement membranes, and alveolar wall tissue. Both collagen and elastin are found in intimate association within the alveolar wall matrix, and form an intricate network of supporting fibres running through the interstitium.[1] Collagen types I and III, in a ratio of approximately 2:1, are the main fibrous components of the interstitium, representing more than 90% of all parenchymal collagens.[2,3] Any alterations in alveolar wall structure are therefore likely to be a consequence of changes in the collagenous composition of the tissue. It has been suggested that the collagens of parenchymal tissue have an important role in lung compliance.[4,5]

Pulmonary emphysema has been defined in pathological terms as "a condition of the lung characterised by abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole, accompanied by destruction of their walls, and without obvious fibrosis."[6] The increase in airspace size with concomitant loss of alveolar wall tissue that occurs in emphysema can be quantified indirectly by measuring airspace wall surface area per unit volume of lung tissue (AWUV). A range of normal AWUV values in relation to age was recently established in a study of the lungs of non-smokers.[7] Current concepts of the pathogenesis of emphysema implicate neutrophil derived proteinases, particularly human neutrophil elastase, as mediators of alveolar wall matrix destruction.[8] Proteinases induce lung

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injury by their proteolytic action on a range of connective tissue proteins including collagens. It is thought that an imbalance between the proteinases released by inflammatory cells and their inhibitors, specifically α-1-proteinase inhibitor, may account for the morphological changes that occur in emphysema.[9,10]

Collagens and elastin are important proteins in emphysema, but attempts to quantify biochemical changes in the amounts of these lung matrix proteins have been confusing, with many studies presenting conflicting data. Most of this work has focused on elastin in emphysema.[11–13] Studies of collagen in emphysema have been few. The characteristic thinning of alveolar wall tissue with increased lung compliance and loss of recoil has been regarded solely as a consequence of alterations in elastin metabolism. Increased alveolar wall collagen (per dry weight, corrected for lung volume[14]) has been more closely associated with inflammatory diseases such as interstitial pulmonary fibrosis (cryptogenic fibrosing alveolitis).

Studies of collagen in emphysema have related biochemical estimations to wet or dry weight of tissue. Recently Cardoso and colleagues[15] showed that, in samples from the lesions of lungs with irregular emphysema, amounts of collagen were raised beyond normal when expressed as μg hydroxyproline/mg freeze dried tissue. We feel, however, that to recognise any change from normality within parenchymal tissue biochemical estimations should be related to a measurement of lung structure such as AWUV. Furthermore, in view of the observations that the airspace walls are the sites of tissue loss in emphysema, biochemical measurements of collagen should be confined only to this alveolated portion of the lung.

We have recently quantified the collagen and elastin content of parenchymal tissue in lungs from smokers with no evidence of macroscopic or microscopic emphysema in multiple $5 \times 5 \times 2$ mm (50 mm^3) samples taken from nine different lungs[16,17] and found that, as the AWUV for the 50 mm^3 samples decreased, the concentrations of both collagen and elastin increased. The relative increase in collagen and elastin was apparent when expressed both per unit volume of lung sample or per unit surface area of alveolar wall. These results suggest either low levels of fibrosis occurring within the tissue, perhaps in response to cigarette smoking, or alternatively anatomical differences in extracellular matrix content according to the portion of the acinar unit contained within the sample. This work also indicated that the approach of combining morphometric measurements of lung structure with quantitative biochemistry is a sensitive way of detecting differences in the extracellular matrix content of the alveolar wall.

In this study we have investigated the effect of both age and microscopic emphysema on the collagen content of alveolar walls in regions of tissue from emphysematous lungs sampled from non-involved areas without any obvious macroscopic lesion. Sampling from these regions may indicate the possible changes in the amounts of alveolar wall collagen occurrin early stage emphysema.

Methods

SELECTION AND CLASSIFICATION OF LUNG SAMPLES

Samples comprised either whole lungs or lo from 59 individuals. Lungs were obtai either at necropsy or from patients undergo surgery for the removal of peripheral l tumours.

Macroscopic emphysema was conside present if airspace size was greater than 1 in diameter on inspection of the mid sag slice from the lung or lobe.[18] Lungs w assessed by an experienced pathologist (D Macroscopic emphysema was classi according to its distribution within the aci as (1) centriacinar emphysema (CAE) – space enlargement around the respirat bronchioles with parenchymal tissue s rounding CAE lesions appearing normal to naked eye; (2) panacinar emphysema (PAE airspace enlargement affecting the whole nar unit; and (3) mixed emphysema (MIX both CAE and PAE lesions present in the sa specimens.[19]

Microscopic emphysema was considered be present if the mean AWUV of the specir was lower than the range of age related AW values recently described in non-smokers. the emphysematous lungs both morphome and biochemical analyses were carried out regions both remote from any lesion and void of macroscopic emphysema.

Lungs were included in the control gr only if clinical records stated that the subj were "life long non-smokers." Twenty th lungs were from life long non-smokers and were from smokers with either no emphyse CAE, PAE, or MIX. The non-smokers cluded lungs from patients aged 22–82 ye while the age ranges of the smoking gro were significantly smaller (table 1). To prev the introduction of bias in the collagen c parisons the non-smokers were age matc with the different smoking groups. The n smoker group contained a number of necrc lungs from young individuals (age range 32; n = 8). These lungs were excluded f any comparisons with the smoking gro Further details of the ages of the non-smo and each of the different smoking groups shown in table 1.

Table 1 Smoking histories and age ranges of the non-smoking and smoking groups investigated

Smoking history	*Age range (years)*	*n*
Non-smokers	22–32	8
	49–82	15
Smokers		
CAE	43–72	13
PAE	57–70	9
MIX	51–68	10
No emphysema	57–70	4

CAE = centriacinar emphysema; PAE = panacinar emphy
MIX = lungs having a mixture of both panacinar and c
acinar emphysema.

LUNG INFLATION

Lobes were floated in a plastic specimen box containing buffered formalin (10%) and inflated by intrabronchial perfusion with buffered formalin at a pressure of 25 cm H_2O from an elevated reservoir until the pleural surface became firm and smooth.[20]

MORPHOMETRY: ESTIMATION OF MEAN LUNG AWUV

Following complete fixation for at least 24 hours the lungs were placed on a purpose built template and 1 cm thick parasagittal slices were taken from each lobe. From the mid sagittal slice at least six random blocks, $20 \times 20 \times 5$ mm, were taken per lobe.[21] The blocks were processed and embedded in glycol methacrylate resin and 3 μm sections were cut from each block and stained by the haematoxylin and eosin method. For each block AWUV was estimated using an automated image scanning system, the fast interval processor (FIP).[16 17 21] Mean AWUV from either six or 12 blocks (depending upon number of lobes available per lung) was calculated for each specimen. AWUV was also estimated in blocks adjacent to the samples prepared for total collagen estimations as described below.

PREPARATION OF TISSUE FOR TOTAL COLLAGEN ESTIMATION

The following technique was devised to enable quantitative biochemistry of collagen amounts to be related to morphometric measurements of lung structure on adjacent tissue sections. The technique was a modification of the method of Lang *et al*[17] devised to quantify collagen and elastin amounts in localised zones of lung tissue inflated with agarose. From the mid sagittal slice of each lobe prepared for mean AWUV estimation (described above) an additional $20 \times 20 \times 5$ mm block was taken at random from the non-smoking and smoking lung groups and stored in 10% formalin. In emphysematous lungs (CAE, PAE, and MIX) all samples were taken from macroscopically non-involved areas away from any lesion. These blocks were removed from the formalin fixative, placed on an absorbent tissue, and gentle pressure applied until the formalin was expelled from the block. The tissue was then placed into a medium sized peel away mould (Cat. No. 18646B, Park Scientific, UK) and fully immersed in approximately 5 ml of optimal cutting temperature cryoembedding compound (OCT Tissue-Tek; Bayer Diagnostics, UK). The block was left in OCT for 15 minutes until the airspaces were fully penetrated. OCT maintains tissue inflation and block rigidity during subsequent histological processing. The blocks were frozen by placing the tissue on a cryostat chuck and immersing the chuck in liquid nitrogen. Each block was left for 10 minutes to equilibrate in the cryostat chamber. In order to measure collagen amounts within known volumes of distended lung tissue, four serial 25 μm sections (total volume 40 mm^3) were taken from each block and placed directly into glass hydrolysis tubes. A further 3 μm section was prepared from each block for AWUV measurement. Details of the procedure are shown in diagrammatic form in fig 1.

MEASUREMENT OF ALVEOLAR WALL COLLAGEN CONTENT

Total collagen was measured (as hydroxyproline) in each 40 mm^3 sample by selective determination of secondary amino acids, using 9-fluoroenylmethylchloroformate (FMOC-Cl) derivatisation.[22] Samples were hydrolysed in liquid phase 6N hydrochloric acid for 24 hours at 110°C, dried in a Speed Vac Concentrator (Savant Instruments, Farmingdale, New York, USA) and reconstituted in 200 μl 0·025% K_3EDTA. High pressure liquid chromatography (HPLC) analysis was performed on a 4·6 mm internal diameter × 25 cm Dynamax-300A column (Rainin Instrument Co, Woburn, Massachusetts, USA) packed with 5 μm C18 bonded spherical silica using a Gilson model 306 solvent delivery system and a model 231 auto sampler (Gilson Medical Electronics SA, Villiers-le-Bel, France). Details of the hydroxyproline quantitation have been described fully by Lang *et al*.[17]

CALCULATION OF THE AMOUNTS OF ALVEOLAR WALL COLLAGEN

In the frozen sectioned material collagen was quantified in a known volume of airspace wall tissue (40 mm^3), and AWUV (mm^2/mm^3) was measured on the adjacent section of tissue. Collagen content is expressed either as hydroxyproline per unit volume ($nmol/mm^3$) or hydroxyproline per unit surface area of airspace wall ($nmol/mm^2$), the latter obtained by dividing the amount per unit volume by the AWUV value for the adjacent slice. In the non-smokers hydroxyproline amounts per unit area of alveolar wall were plotted against mean case AWUV, the latter being determined from the glycol methacrylate embedded blocks.

HYDROXYPROLINE AND FORMALIN FIXATION

By normalising our collagen amounts to local lung structure we were able to eliminate any possible variations in shrinkage with length of storage in fixative.

We are unaware of any effect of formalin fixation on the determination of hydroxyproline. Control experiments on lung samples from different regions of the same lung, with or without formalin fixation, showed no significant difference in collagen amounts per unit volume (data not shown). Furthermore, the storage times of the various experimental groups were similar, hence any long term effects of formalin fixation would apply equally to all groups.

STATISTICAL ANALYSIS

In the non-smoking group the effects of ageing and mean lung AWUV on the hydroxyproline

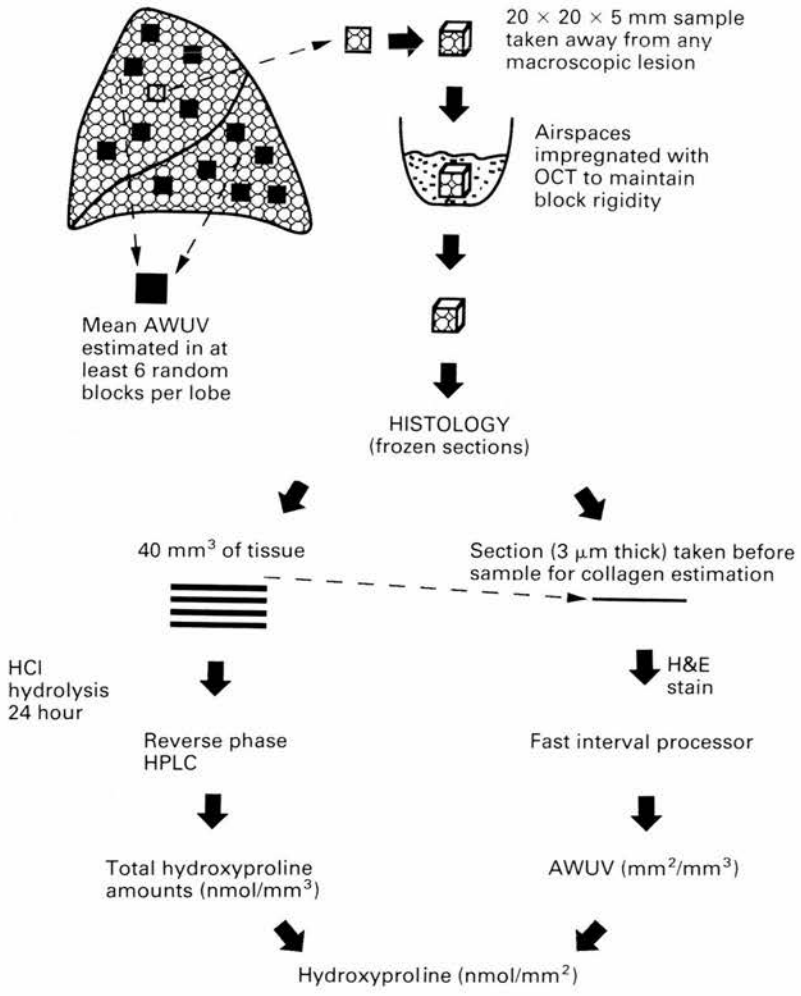

Figure 1 Schematic representation of the method of sample preparation of alveolar wall tissue for morphometric measurement of AWUV and biochemical estimations of collagen.

content of the alveolar wall tissue were compared by multivariate regression analysis.[23] Hydroxyproline data of the lungs of smokers were compared with those of non-smokers using the non-parametric rank Mann-Whitney U test for the difference between the group medians. Median and mean (SE) values are shown for the amounts of hydroxyproline, patient age, and lung AWUV. Differences were considered significant if $p < 0.05$.

Results

MEAN LUNG AWUV VALUES

Non-smokers

In the non-smokers the mean lung AWUV dropped significantly with age from 22·34 to 14·02 mm^2/mm^3 (age range 22–82 years; $r = -0.801$, $p < 0.001$). A similar age difference has previously been shown in a larger sample of lungs from non-smokers.[7]

Smokers

The lungs of smokers assessed to be free of any macroscopic emphysema had mean AWUV values within the normal age related limits in non-smokers (table 2). The median AWUV values of each group of macroscopic emphysematous lungs (CAE, PAE, and M were significantly lower than the median the non-smoking group (table 2). When in vidual lungs within each group were c sidered in relation to the lower limit of norm ity of the non-smoking population we fo that, of the 13 CAE lungs measured two mean AWUV values below normal, whil the nine PAE lungs five had lower AW values than normal, and in the MIX group of the 10 lungs were abnormal. In a la sample of emphysematous lungs[24] mean l AWUV values in 15% of CAE lungs v below the lower limit of normality, wherea PAE and MIX lungs 63% and 58% resp ively were below the normal limit (Gill and Lamb, unpublished observations).

ALVEOLAR WALL COLLAGEN CONTENT

Non-smokers

In the lungs of non-smokers collagen cont expressed as hydroxyproline per unit vol (fig 2A) or per unit surface area (fig 2B alveolar wall tissue showed no significant relation with case AWUV. The ranges of l roxyproline content in the alveolar wall ti

Table 2 Median, mean (SE) lung AWUV and hydroxyproline content of alveolar wall tissue (expressed per unit volume and per unit surface area) of the smoking and non-smoking groups

Smoking history	*Median and mean age (years)*	*Median and mean AWUV (mm^2/mm^3)*	*Median and mean hydroxyproline (nmol/mm^3)*	*Median and mean hydroxyproline (nmol/mm^2)*
Non-smokers	63·0, 64·3 (3·30)	17·06, 17·23 (0·50)	4·62, 4·56 (1·44)	0·254, 0·272 (0·026)
Smokers				
CAE	62·0, 59·3 (2·71)	15·91, 16·01 (0·69)*	5·80, 7·18 (1·10)	0·371, 0·438 (0·058)*
PAE	62·0, 62·2 (3·16)	10·45, 12·23 (1·25)**	7·68, 8·11 (3·01)**	0·461, 0·643 (0·169)**
MIX	63·0, 62·4 (1·69)	11·45, 12·58 (1·10)**	6·63, 6·71 (3·79)*	0·676, 0·716 (0·068)**
No emphysema	66·0, 64·8 (3·01)	17·01, 17·30 (0·97)	5·82, 5·48 (1·75)	0·319, 0·343 (0·058)

CAE = macroscopically assessed centriacinar emphysema; PAE = macroscopically assessed panacinar emphysema; MIX = mixture of both panacinar and centriacinar emphysema.
* $p < 0.05$, ** $p < 0.005$ smoking group *v* non-smoking group.

of the lungs of non-smokers (n = 23 samples, age range 22–80 years) were 2·46–8·03 nmol/mm^3 (per unit volume) and 0·116–0·515 nmol/mm^2 (per unit surface area). Furthermore, when patient age was considered we found no significant relation with alveolar wall collagen content, either per unit volume (fig 2C) or per unit surface area (fig 2D). When age, mean lung AWUV, and hydroxyproline content of the alveolar wall were compared by multivariate regression analysis hydroxyproline was not significantly related to either age or mean lung AWUV.

Smokers

Table 2 shows data obtained from the lungs of smokers for patient age, mean lung AWUV, and collagen content expressed per unit volume and per unit surface area of alveolar wall tissue. Patient ages in each of the smoking groups were not statistically different from the older subpopulation of non-smokers (table 1). In the PAE and MIX groups there were significantly increased amounts of collagen compared with the non-smokers when expressed both per unit volume or per unit surface area of airspace wall tissue. Collagen amounts were also significantly raised in CAE lungs when expressed per unit surface area of alveolar wall tissue. However, when collagen was expressed per unit volume CAE lungs showed no significant change from the lungs of non-smokers. Table 2 shows the significance values of the differences in alveolar wall collagen content between the various smoking groups and the

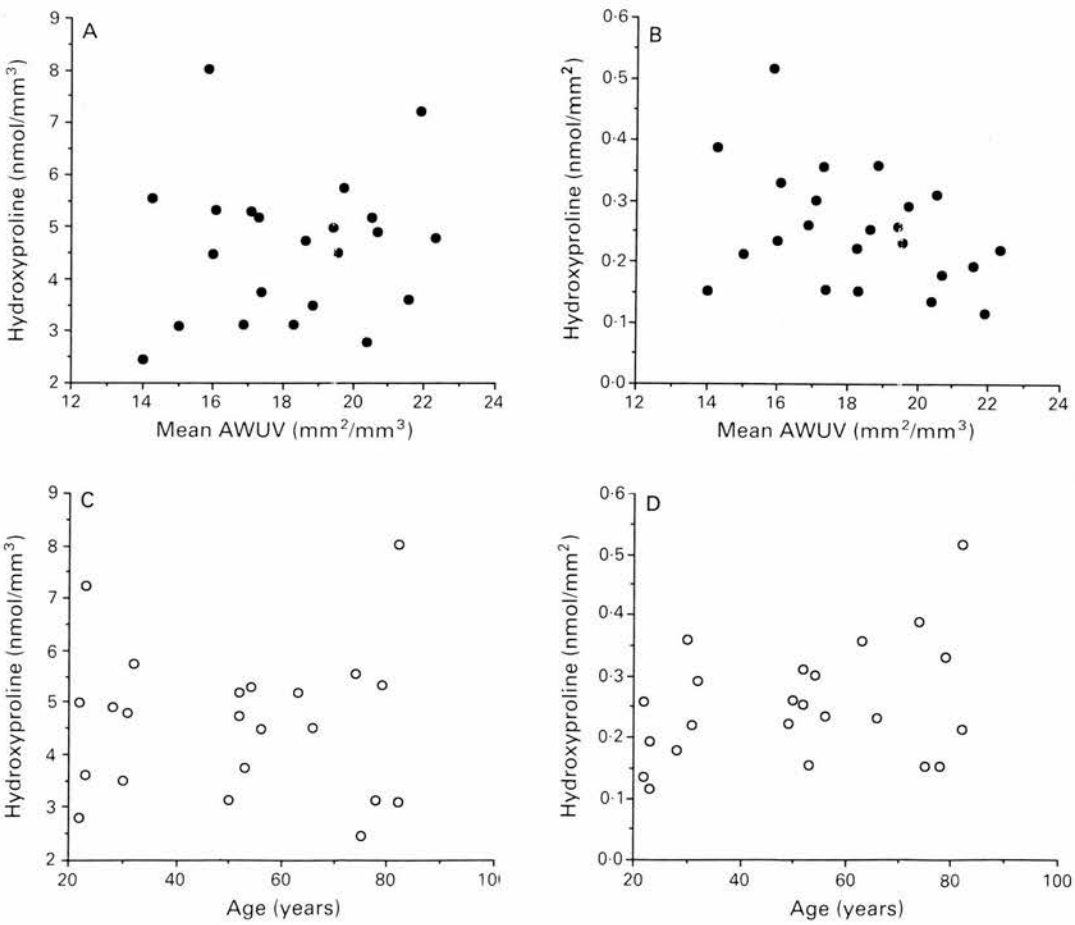

Figure 2 (A) Mean lung AWUV in 23 life long non-smokers plotted against alveolar wall collagen content (as hydroxyproline) per unit volume. (B) Mean lung AWUV in 23 life long non-smokers plotted against alveolar wall collagen content per unit surface area of alveolar wall. (C) Alveolar wall collagen (hydroxyproline) content per unit volume plotted against patient age in non-smokers. (D) Collagen in the lungs of non-smokers expressed per unit surface area of alveolar wall tissue plotted against patient age.

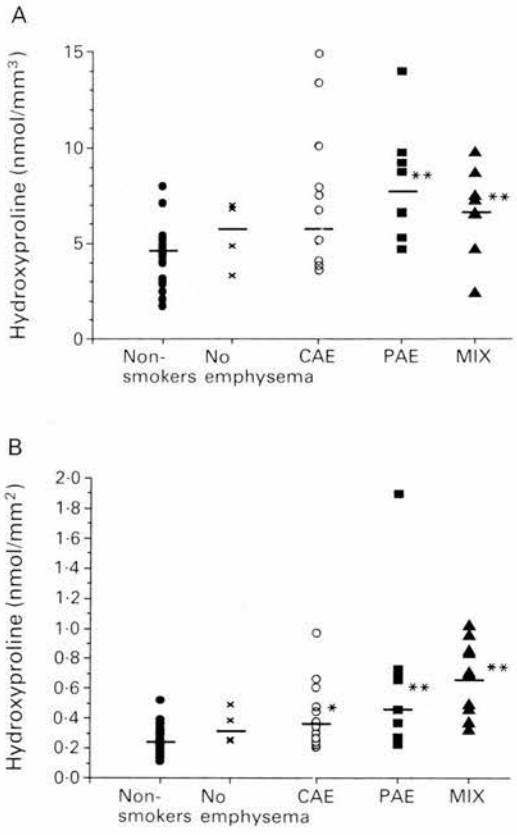

*Figure 3 (A) Median alveolar wall collagen content (as hydroxyproline per unit volume alveolar wall tissue) in the lungs of smokers (with and without macroscopic emphysema) compared with life long non-smokers. CAE = centriacinar emphysema; PAE = panacinar emphysema; MIX = both CAE and PAE. Data analyses were made on similar age groups. In the CAE, PAE, and MIX lungs samples were taken from regions showing no macroscopic emphysema. For each group median collagen content is represented by a horizontal bar. Significant differences from the non-smoking group are indicated: * $p < 0{\cdot}05$, ** $p < 0{\cdot}005$. (B) As (A) with collagen expressed per unit surface area of alveolar wall tissue. Significant differences from the non-smoking group are indicated: * $p < 0{\cdot}05$, ** $p < 0{\cdot}005$.*

age matched non-smoking group. Figures 3A and B show the collagen contents of the lungs of individual non-smokers and smokers. There was an increased scatter in the data for CAE, PAE, and MIX lungs compared with both the non-smokers and the smokers without emphysema.

Discussion

In this study we have quantified airspace structure morphometrically in emphysema and related our measurements to biochemical estimations of collagen content. Emphysema was classified into subtypes depending upon the distribution of abnormal airspace within the acinar unit. Analyses of collagen content in emphysematous lungs were performed in areas with minimal macroscopic abnormality. Assessment of microscopic emphysema[24] may identify mild microscopic PAE not visible macroscopically. However, for the purposes of this paper we have confined our classification to macroscopically assessed emphysema.

We have used a morphometric measurement – the amount of airspace wall surface area per unit volume (AWUV) – to assess lung structure. The use of AWUV enables the detect of early stage microscopic enlargement of spaces. Amounts of collagen in the alve wall tissue can then be expressed either unit volume or per unit surface area. To knowledge this is the first investigation re ing biochemical analyses of amounts of co gen in airspace tissue to detailed measu ments of local and mean lung architecture both non-emphysematous and emphyse tous lungs.

COLLAGEN IN NORMAL, NON-SMOKERS' LUNG

As the normal lung ages, airspace size creases with an associated loss of AWU Biochemical estimations must therefore t into account this change with age. In orde identify alterations in connective tissue p teins in emphysema statistical comparis must be made between samples of compara ages.

In view of the loss in alveolar wall tis density with age, we may speculate that th could also be age related changes in the cont of extracellular matrix. Our observatio however, showed no significant correlat between the collagen content of the alve wall and either increasing age or change mean lung AWUV when collagen expressed per unit volume of alveolar w tissue. When collagen amounts were expres per unit surface area of alveolar wall tis correlations with both change in age and m lung AWUV were apparent, but these w not statistically significant. It should be no that we have calculated mean lung AWU from at least six random blocks per lung, wh hydroxyproline per unit surface area is deri from AWUV measured on slices adjacent those prepared for collagen determinatio The data indicate that, although there i significant loss of alveolar wall tissue as human lung ages, there may be an act process maintaining a constancy in amount structural collagen that enable the lung function.

COLLAGEN IN SMOKERS' LUNGS

To identify changes from normal in emphy matous lung collagen we have expres amounts both volumetrically and per unit s face area. These data may not be dire comparable to work published elsewhere. example, amounts of collagen have often b reported per dry weight.[14,15] As our intentio to relate alveolar wall collagen to change regional lung structure we feel that a m meaningful normalisation of the collagen c is either to volume or alveolar wall sur area. When expressed per unit surface are alveolar wall the effects of any local differe in lung inflation are automatically elimina

The lungs of smokers without macrosc emphysema had mean AWUV values wi the normal range for age.[7] Although the al lar wall collagen content of these lungs ten to be higher than in the lungs of non-smok the difference was insignificant, perh

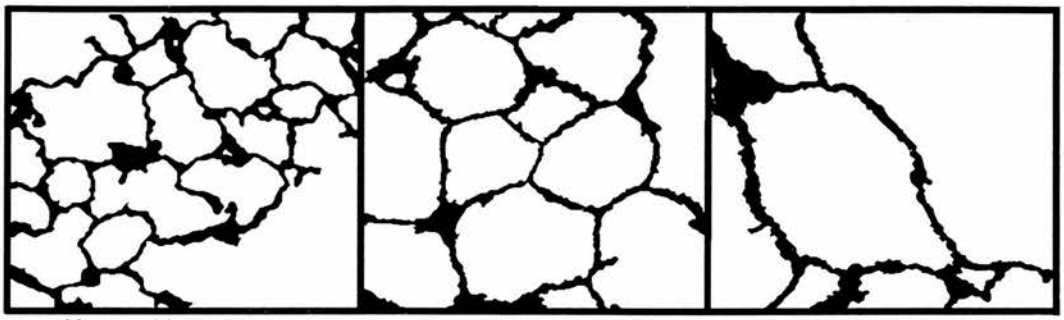

Figure 4 Digital binary image demonstrating the enlargement of airspace size and concomitant loss of alveolar wall, both with age (22 and 82 year old non-smokers) and in a lung from a 70 year old smoker with panacinar emphysema (PAE). In the PAE lung airspace enlargement due to microscopic emphysema can be seen. Magnification × 40.

because of the small sample size (n = 4; table 2). A previous study of smokers' lungs free of any macroscopic or microscopic emphysema clearly demonstrated increased amounts of alveolar wall collagen and elastin (either per unit volume or per unit surface area) in samples with low AWUV compared with the more alveolated specimens which contained relatively less collagen and elastin.[16 17] These earlier analyses were made in 5 × 5 × 2 mm samples and may have identified variations in AWUV within the acinar unit.

In emphysematous lungs we may expect an increase in airspace size with concomitant loss of alveolar wall surface area, as stated in the definition of emphysema.[6] For example, fig 4 demonstrates the extent of microscopic airspace enlargement in two lungs from an 82 year old non-smoker and a 70 year old smoker with PAE compared with a 22 year old life long non-smoker. From such morphological changes we might expect a loss, or at least no change, in amounts of collagen per unit volume of lung. Surprisingly, however, when the lungs of smokers with emphysema were compared as a group to the lungs of non-smokers of similar ages, the emphysematous group contained significantly more collagen per unit volume (median hydroxyproline content 4·49 nmol/mm^3 in non-smokers *v* 6·62 nmol/mm^3 in emphysematous lungs). Similarly, when the amounts of collagen in each emphysematous group were considered individually in comparison with the lungs of life long non-smokers (age ranges 49–82 years), collagen amounts per unit volume were also elevated beyond normal in each group except CAE. When expressed per unit surface area of alveolar wall the amount of collagen increased in all emphysematous groups including CAE. In lungs with CAE the median alveolar wall collagen per unit surface area was increased some 1·6 times, whereas both PAE and MIX lungs had an increase in collagen content per unit surface area of 2·4–2·5 times respectively.

A recent study[15] found that collagen (as a proportion of birefringence per volume of sample) appeared to be increased within lesions from CAE, distal acinar, and irregular airspace enlargement. However, a biochemical increase in collagen (as hydroxyproline μg/mg of tissue) was only detected in lesions from lungs with irregular airspace enlargement. Our results on non-macroscopically involved regions of alveolar wall tissue indicate that collagen per unit volume remains unchanged in CAE, but in PAE the amounts are significantly raised despite reduced amounts of alveolar wall per unit surface area. Furthermore, morphometric measurements of airspace wall have shown that around 85% of CAE lungs have mean lung AWUV values within normal limits for age.[24] This observation may highlight the larger proportion of normal, uninvolved regions surrounding the emphysematous lesions in CAE which may contain less fibrotic airspace wall than PAE lungs in which airspace enlargement has affected the whole acinar unit. This finding lends further support to the hypothesis that there may be alternative pathogenetic mechanisms contributing to the morphometric and biochemical differences in lungs with purely CAE or PAE.

EMPHYSEMA AND COLLAGEN METABOLISM

There have been few previous studies of the changes in the amount of collagen in patients with emphysema. Emphysema is usually regarded as a condition of the lung in which elastin amounts are altered, although data on this are conflicting with reports of either no change,[12] or decreased amounts[13 15] of elastin. Changes in collagen have been more closely associated with inflammatory diseases such as idiopathic pulmonary fibrosis (cryptogenic fibrosing alveolitis) in which increases in the total collagen have suggested a progressive deposition of collagen throughout the disease.[14]

Normal lung collagen homeostasis requires that collagen synthesis be counterbalanced by mechanisms in which collagen is degraded.[25 26] A tight balance between synthesis and degradation of connective tissue proteins therefore enables tissue which is rich in collagens, such as alveolar wall, to function normally. Our data indicate that in emphysema (CAE, PAE, and MIX lungs) there is likely to be an imbalance between the two processes which leads to an increase or accumulation of collagen mass within the tissue of the alveolar walls. Turnover studies are required to determine

whether the observed increase in collagen in emphysema is due to increased synthesis, decreased degradation, or both.

The definition of emphysema states an increase in airspace size but "without obvious fibrosis." In this paper the amounts of collagen represent an increase beyond normal in macroscopically "normal" alveolar wall regions of emphysematous lungs. Despite the definition of emphysema[6] there appears to be a clear association between emphysema and an increase in the collagen content (fibrosis) of the alveolar wall tissue detectable at the level of quantitative biochemistry.

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